

NOVEL NUCLEIC ACID PROBES, METHOD FOR DETERMINING  
CONCENTRATIONS OF NUCLEIC ACID BY USING THE PROBES,  
AND METHOD FOR ANALYZING DATA OBTAINED BY THE METHOD

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BACKGROUND OF THE INVENTION

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determine a concentration of a nucleic acid by using a nucleic acid probe labeled with a fluorescent dye. These methods include:

(1) Dot blotting assay

After a target nucleic acid and a nucleic acid probe labeled with a fluorescent dye are hybridized on a membrane, unreacted nucleic probe is washed off. The intensity of fluorescence only from fluorescent dye molecules, by which the nucleic acid probe hybridized with the target nucleic acid is labeled, is measured.

(2) Method making use of an intercalator: Glazer et al., Nature, 359, 959, 1992

A certain specific fluorescent dye called "intercalator" emits strong fluorescence upon its insertion into a double strand of a nucleic acid. This method measures an increase in fluorescence from the fluorescent dye. Examples of the fluorescent dye can include ethidium bromide [Jikken Igaku (Laboratory Medicine), 15(7), 46-51, Yodosha (1997)] and SYBR R Green I (LightCycler™ System, April 5, 1999; pamphlet distributed by Roche Diagnostics, Mannheim, Germany).

(3) Method making use of FRET (fluorescence energy transfer):

Mergny et al., Nucleic Acid Res., 22, 920-928, 1994

This method comprises hybridizing two nucleic acid probes to a target nucleic acid. These two nucleic acid probes are labeled by different fluorescent dyes, respectively. The

fluorescent dye of one of the two probes can transfer energy to the fluorescent dye of the other probe such that the latter fluorescent dye is caused to emit fluorescence. These two probes are designed such that they hybridize with their fluorescent dyes being located opposite each other and apart from each other by 1 to 9 bases. When these two nucleic acid probes hybridize to the target nucleic acid, emission of fluorescence from the latter fluorescent dye takes place. The intensity of this fluorescence emission is proportional to the number of replications of the target nucleic acid.

(4) Molecular beacon method: Tyagi et al., *Nature Biotech.*, **14**, 303-308, 1996

A nucleic acid probe for use in this method is labeled at an end thereof with a reporter dye and at an opposite end thereof with a quencher dye. As both end portions of the probe are complementary with each other in their base sequences, the overall base sequence of the probe is designed to form a hairpin stem. Owing to this structure, emission from the reporter dye is suppressed by the quencher dye under Forster resonant energy transfer in a state suspended in a liquid. When the probe hybridizes to a target nucleic acid, the hairpin stem structure is broken. This leads to an increase in the distance between the reporter pigment and the quencher pigment, so that the transfer of Forster resonant energy no longer takes place. This allows the reporter dye to make emission.

(5) Davis's method: Davis et al., Nucleic Acids Res., 24, 702-706, 1996

This method uses DNA constructs containing one or two fluorescein molecules in flow cytometry. The fluorescein molecules were attached to the 3' end of a DNA probe through an 18-atom spacer arm that resulted in a 10-fold increase in fluorescence intensity compared to the DNA probe to which fluorescein was directly attached to the 3' end of the probe.

Applied to various determination methods for nucleic acids, Fish methods (fluorescent in situ hybridization assays), PCR methods, LCR methods (ligase chain reactions), SD methods (strand displacement assays), competitive hybridization and the like, significant developments have been made on these methods.

(6) Substantial technical improvements have been made on methods for amplifying a target gene by PCR [Tanpakushitsu, Kakusan, Koso (Proteins, Nucleic Acids, Enzymes), 35(17), KYORITSU SHUPPAN CO., LTD. (1990)] and conducting a polymorphous analysis on the target gene so amplified, and these polymorphous analysis methods have now found wide-spread utility in various fields such as medical field [Jikken Igaku (Laboratory Medicine), 15(7), Yodosha (1997)]. Various diseases, especially immune-related diseases have hence been elucidated from genes, thereby obtaining certain successful outcomes.

Although these methods are now widely used, they include a disadvantageous step that, subsequent to hybridization reaction between a nucleic acid probe labeled with a fluorescent dye and a target nucleic acid, an unhybridized portion of the nucleic acid probe has to be washed out of the reaction system. Obviation of this step can apparently bring about shorter determination time, simplified determination, and accurate determination. There is, accordingly, a long-standing desire for the development of a nucleic acid determination method which does not include such a step.

#### SUMMARY OF THE INVENTION

With the foregoing in view, the present invention has as an object thereof the provision of a method for determining a concentration of a target nucleic acid by using a nucleic acid probe labeled with a fluorescent dye, which makes it possible to determine the concentration of the target nucleic acid in a shorter time, more easily and more accurately, and also the provision of nucleic acid probes useful for the practice of the method and various devices making use of the probes.

The present invention also has as a second object thereof the provision of a novel polymorphous analysis method for easily and quickly performing determination of a polymorphous composition of a target gene and reagent kits useful in the method, a computer-readable recording medium with programmed

procedures, which are required to make a computer perform a method for analyzing data obtained by the quantitative polymorphous analysis method, and an analysis system for the quantitative polymorphous analysis.

5 To achieve the above-described objects, the present inventors have proceeded with a variety of investigations and have obtained findings as will be described below.

10 A detailed study was conducted on a variety of nucleic acid probes, and in a trial and error manner, many probes were prepared. As a result, it has been found that, even in the case of a nucleic acid probe composed of an oligonucleotide which does not form a stem-loop structure between nucleotide chains at positions where the oligonucleotide is labeled with a fluorescent dye and a quencher substance, respectively, labeling by the dye and substance at specific positions may allow the quencher substance to act on the emission of fluorescence from the fluorescent dye and may give quenching effect on the emission of fluorescence.

15 The present inventors have proceeded with an investigation on methods for determining a concentration of a nucleic acid by using a nucleic acid probe. As a result, it was found that emission of fluorescence from a fluorescent dye decreases (quenching phenomenon of fluorescence) when a nucleic acid probe labeled with the fluorescent dye hybridizes to a target nucleic acid. It was also found that this decrease is

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significant with certain specific dyes. It was also found that the extent of this decrease varies depending on bases in a probe portion, to which the fluorescent dye is conjugated, or on the sequence of the bases.

- 5     ③     Performance of a polymorphous analysis on a target gene after amplifying the target gene by a quantitative gene amplification method makes it possible to easily and quickly determine the pre-amplification amount and polymorphous composition of the target gene with good quantitateness.

10           The present invention has been completed based on the above-described findings.

          Therefore, the present invention provides the following (novel) nucleic acid probes, methods, kits and devices:

- 15     1)     A novel nucleic acid probe for determining a concentration of a target nucleic acid, comprising:

          a single-stranded oligonucleotide capable of hybridizing to the target nucleic acid, and

          a fluorescent dye and a quencher substance, both of which are labeled on the oligonucleotide,

20           wherein the oligonucleotide is labeled with the fluorescent dye and the quencher substance such that an intensity of fluorescence in a hybridization reaction system increases when the nucleic acid probe is hybridized with the target nucleic acid; and the oligonucleotide forms no stem-loop structure between bases at positions where the

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oligonucleotide is labeled with the fluorescent dye and the quencher substance, respectively.

2) A nucleic acid probe for determining a concentration of a target nucleic acid, the probe being labeled with a fluorescent dye, wherein:

the probe is labeled at an end portion thereof with the fluorescent dye, and

the probe has a base sequence designed such that, when the probe hybridizes at the end portion thereof to the target nucleic acid, at least one G (guanine) base exists in a base sequence of the target nucleic acid at a position 1 to 3 bases apart from an end base of the target nucleic acid hybridized with the probe;

whereby the fluorescent dye is reduced in fluorescence emission when the probe labeled with the fluorescent dye hybridizes to the target nucleic acid.

3) A nucleic acid probe for determining a concentration of a target nucleic acid, the probe being labeled with a fluorescent dye, wherein:

the probe is labeled at an end portion thereof with the fluorescent dye, and

the probe has a base sequence designed such that, when the probe hybridizes to the target nucleic acid, plural base pairs in a probe-nucleic acid hybrid complex form at least one G (guanine) and C (cytosine) pair at the end portion;

whereby the fluorescent dye is reduced in fluorescence emission when the probe labeled with the fluorescent dye hybridizes to the target nucleic acid.

4) A nucleic acid probe for determining a concentration of a target nucleic acid, the probe being labeled with a fluorescent dye, wherein:

the probe is labeled at a modification portion other than a 5' end phosphate group or a 3' end OH group thereof with the fluorescent dye, and

the probe has a base sequence designed such that, when the probe hybridizes to the target nucleic acid, plural base pairs in a probe-nucleic acid hybrid complex form at least one G (guanine) and C (cytosine) pair at the modification portion;

whereby the fluorescent dye is reduced in fluorescence emission when the probe labeled with the fluorescent dye hybridizes to the target nucleic acid.

5) A nucleic acid probe as described above under any one of 1) to 4) for determining a concentration of a nucleic acid, wherein the oligonucleotide of the nucleic acid probe for the measurement of the nucleic acid is a chemically-modified nucleic acid.

6) A nucleic acid probe as described above under any one of 1) to 5) for determining a concentration of a target nucleic acid, said nucleic acid probe being labeled with a fluorescent dye, wherein the oligonucleotide of the nucleic acid probe for

the determination of the nucleic acid is a chimeric oligonucleotide which comprises a ribonucleotide and a deoxyribonucleotide.

7) A method for determining a concentration of a target nucleic acid, which comprises:

hybridizing a nucleic acid probe as described above under any one of 1) to 6) to the target nucleic acid, and measuring an intensity of fluorescence in a measuring system.

8) A method for determining a concentration of a target nucleic acid, which comprises:

hybridizing a nucleic acid probe as described above under any one of 1) to 6) to the target nucleic acid, and measuring a change in fluorescence emission from the fluorescent dye after the hybridization relative to fluorescence emission from the fluorescent dye before the hybridization.

9) A method for determining a concentration of a target nucleic acid by using a nucleic acid probe as described above under any one of 1) to 6), wherein the nucleic acid probe and the target nucleic acid are hybridized to each other after subjecting the target nucleic acid to heat treatment under conditions suited for sufficient degradation of a high-order structure of the target nucleic acid.

10) A method as described above under 9) for measuring a

concentration of a target nucleic acid, wherein a helper probe for the practice of a hybridization reaction is added to a hybridization reaction system before the hybridization reaction.

- 5 11) A method for analyzing or determining polymorphism and/or mutation of a target nucleic acid, which comprises:

hybridizing a nucleic acid probe as described above under any one of 1) to 6) to the target nucleic acid, and measuring a change in an intensity of fluorescence.

- 10 12) A novel quantitative, polymorphous analysis method comprising:

amplifying a target gene by a quantitative gene amplification method; and

- 15 performing a polymorphous analysis with respect to the target gene to determine an amount of the target gene and a polymorphous composition or amounts of individual components of the target gene.

- 20 13) A quantitative, polymorphous analysis method as described above under 12), wherein the polymorphous analysis is T-RELP (terminal restriction fragment length polymorphism), RFLP (restriction fragment length polymorphism), SSCP (single strand conformation) or CFLP (cleavage fragment length polymorphism).

- 25 14) A quantitative, polymorphous analysis method as described above under 12) or 13), wherein the quantitative gene

amplification method is quantitative PCR or real-time monitoring quantitative PCR.

15) A kit for determining a concentration of a target nucleic acid, wherein the kit includes or is accompanied by a nucleic acid probe as described above under any one of 1) to 6) or a nucleic acid probe and a helper probe as described above under any one of 1) to 6).

16) A kit for analyzing or determining polymorphism and/or mutation of a target nucleic acid, comprising a nucleic acid probe as described above under any one of 1) to 6) or a nucleic acid probe and a helper probe as described above under any one of 1) to 6).

17) A reagent kit for use in quantitative PCR, wherein the kit includes or is accompanied by a nucleic acid probe as described above under any one of 1) to 6) or a nucleic acid probe and a helper probe as described above under any one of 1) to 6).

18) A device for determining a concentration of at least one target nucleic acid out of plural nucleic acids, comprising:

a solid support, and

a like plural number of nucleic acid probes as described above under any one of 1) to 6) bound on a surface of the solid support such that the concentration of the target nucleic acid can be determined by hybridizing the target nucleic acid to the corresponding one of the probes and determining a change in an

intensity of fluorescence.

19) A method for determining a concentration of a target nucleic acid, which comprises determining the concentration of the target nucleic acid or analyzing or determining

5 polymorphism and/or mutation of the target nucleic acid by using a nucleic acid determination device as described above under 18), or a quantitative, polymorphous analysis method of a target nucleic acid, which comprises performing a quantitative, polymorphous analysis of the target nucleic acid by using a  
10 nucleic acid determination device as described above under 18).

20) A nucleic acid determination method, a method for analyzing or determining polymorphism and/or mutation of a target nucleic acid, or a quantitative, polymorphous analysis method as described above under any one of 7) to 14), wherein  
15 the target nucleic acid is a nucleic acid contained in cells derived from a microorganism or animal obtained by single colony isolation or a nucleic acid contained in a homogenate of the cells.

21) A method for determining a concentration of a target  
20 nucleic acid by using PCR, which comprises:

conducting reactions in PCR by using a nucleic acid probe as described above under any one of 1) to 6), and

determining an initial concentration of the amplified target nucleic acid from percentage of a change in an intensity  
25 of fluorescence occurred as a result of hybridization between

the probe and the amplified target nucleic acid.

22) A method for determining a concentration of a target nucleic acid by using PCR, which comprises:

conducting reactions in PCR by using as a primer a nucleic acid probe as described above under any one of 1) to 6), and  
5 determining an initial concentration of the amplified target nucleic acid from percentage of a change in an intensity of fluorescence occurred as a result of hybridization between the primer or an amplified nucleic acid amplified from the primer and the amplified target nucleic acid.  
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23) A method for determining an initial concentration of a target nucleic acid amplified in PCR, which comprises:

conducting reactions in PCR by using a nucleic acid probe as described above under any one of 1) to 6);

15 measuring an intensity of fluorescence in a reaction system in which in a course of a nucleic acid extending reaction, the probe has been degraded out by polymerase or in which a nucleic acid denaturing reaction is proceeding or has been completed and also an intensity of fluorescence in the reaction  
20 system in which the target nucleic acid or amplified target nucleic acid is hybridized with the nucleic acid probe; and then

calculating percentage of a change in the latter intensity of fluorescence from the former intensity of fluorescence.

25 24) A method for determining an initial concentration of a

nucleic acid amplified in PCR, which comprises:

conducting reactions in PCR by using, as a primer, a nucleic acid probe as described above under any one of 1) to 6);

5           measuring an intensity of fluorescence in a reaction system in which the probe and the target nucleic acid or amplified nucleic acid have not hybridized with each other and also an intensity of fluorescence in the reaction system in which the probe and the target nucleic acid or amplified nucleic acid are hybridized with each other; and then

10           calculating percentage of a decrease of the former intensity of fluorescence from the latter intensity of fluorescence.

25)   A method as described above under 23) or 24) for  
15   determining a concentration of a nucleic acid amplified in PCR, wherein the PCR is real-time quantitative PCR.

26)   A method for analyzing data obtained by a nucleic acid determination method as described above under any one of 23) to 25), further comprising correcting an intensity value of  
20   fluorescence in a reaction system, said intensity value being available after the target nucleic acid has hybridized to the nucleic acid probe labeled with the fluorescent dye, in accordance with an intensity value of fluorescence in the reaction system available after a probe-nucleic acid hybrid  
25   complex so formed has been denatured.

27) A method for analyzing data obtained by a real-time quantitative PCR method as described above under any one of 23) to 25), further comprising, as a correction processing step, correcting an intensity value of fluorescence in a reaction system, said intensity being available in each cycle after the amplified nucleic acid has conjugated to the fluorescent dye or after the amplified nucleic acid has hybridized to the nucleic acid probe labeled with the fluorescent dye, in accordance with an intensity value of fluorescence in the reaction system available after a nucleic acid-fluorescent dye conjugate or probe-nucleic acid hybrid complex so formed has been denatured in the cycle.

28) A method for analyzing a melting curve of a target nucleic acid, which comprises:

performing PCR on the target nucleic acid by using a nucleic acid probe as described above under to any one of 1) to 6); and

analyzing the melting curve of the target nucleic acid to determine a  $T_m$  value of each amplified nucleic acid.

Numerous advantageous effects have been brought about by the present invention as will be set out below.

1) First aspect of the invention (fluorescence emitting probe)

As the probe according to the present invention has been obtained by simply binding the fluorescent dye and the quencher substance to the single-stranded deoxyribooligonucleotide

which does not form any stem loop, the designing of the base sequence of a probe which hybridizes to a target nucleic acid is not complex and is easy. Further, the emission of fluorescence from the fluorescent dye is suppressed by the quencher substance before the probe hybridizes to the target nucleic acid, so that the background of a measurement is extremely low. Accordingly, the measurement of the target nucleic acid is accurate. Moreover, the measurement is simple and can be conducted in a short time.

2) Second aspect of the invention (fluorescence quenching probe)

(1) The probe according to the present invention has been obtained by simply binding the specific fluorescent dye to the single-stranded deoxyribooligonucleotide. The probe is designed such that the intensity of fluorescence decreases when the reaction system changes from a non-hybridization system to a hybridization system. Therefore, the designing of the probe is not complex and is easy. As a consequence, the measurement of a target nucleic acid is accurate and simple.

(2) In particular, the fluorescence quenching probe according to the present invention, which comprises the chemically-modified oligonucleotide or the like, or the fluorescence quenching probe according to the present invention, which comprises the chimeric oligonucleotide, has been developed for the determination of RNA having a complex

structure, especially a nucleic acid such as tRNA. This invention has made it possible to determine such a nucleic acid easily, simply and accurately.

3) Third aspect of the present invention (the invention relating to use of the above-described fluorescence emitting probe and fluorescence quenching probe according to the present invention)

(1) Use of fluorescence emitting probes or fluorescence quenching probes according to the present invention makes it possible to simply and easily produce a determination kit for determining a concentration of a target nucleic acid, said kit including or being accompanied by such probes, or a nucleic acid chip or nucleic acid device such as a DNA chip with the probes bound thereon.

(2) Since use of the method, determination kit, nucleic acid chip or nucleic acid device according to the present invention does not require an operation such as that needed to remove unreacted nucleic acid probe from a determination system, the concentration of a target nucleic acid can be determined in a short time and with ease.

(3) When applied to a co-cultivation system of microorganisms or a symbiotic cultivation system of microorganisms, the viable count of a particular microorganism strain in the system can be specifically measured in a short time.

(4) Further, the present invention has also made it possible to simplify, with improved accuracy, determination of polymorphism, such as SNP (single nucleotide polymorphism), or mutation of a target nucleic acid.

5 (5) Further, the quantitative PCR method making use of probes of the present invention has the following advantageous effects:

- 10 a. As the quantitative PCR method does not involve addition of any factor which may act in an inhibitive manner on amplification of a target nucleic acid by Taq DNA polymerase, quantitative PCR can be conducted under similar conditions as conventionally-known usual PCR having specificity.
- 15 b. The specificity of PCR can be maintained high, so that amplification of primer dimer is retarded. Compared with conventionally-known quantitative PCR, the quantitation limit can be lowered on the order of about one digit.
- 20 c. It is no longer required to provide a complex nucleic acid probe. It is, therefore, possible to save time and cost which would otherwise be required for such a complex nucleic acid probe.
- 25 d. A target nucleic acid can be effectively amplified, so that the amplification step can be monitored in real time.

(6) The present invention has also provided the method for analyzing data obtained by real-time quantitative PCR which makes use of fluorescence emitting probes or fluorescence quenching probes according to the present invention.

5 (7) The data analysis method according to the present invention can be used to prepare a working line for the determination of the number of copies of a nucleic acid in a nucleic acid sample of unknown nucleic acid copy number. This working line has a correlation coefficient which is far higher than those available by conventional methods. Use of the data  
10 analysis method according to the present invention, therefore, makes it possible to accurately determine the number of copies of nucleic acid.

(8) A working line the correlation efficient of which is high  
15 can be automatically prepared by the use of the data analysis software relating to the analysis method of data obtained by real-time quantitative PCR, the computer-readable recording medium with the procedures of the analysis method recorded as a program therein, or the determination or analysis system for  
20 the real-time quantitative PCR. The data analysis software, computer-readable recording medium, and the determination or analysis system all pertain to the present invention.

(9) Further, use of the novel method according to the present invention for the analysis of the melting curve of a nucleic  
25 acid makes it possible to determine the  $T_m$  value of the nucleic

acid with high accuracy. Moreover, use of the data analysis software for the method, the computer-readable recording medium with the procedures of the analysis method recorded as a program therein, or the determination or analysis system for the real-time quantitative PCR makes it possible to obtain an accurate  $T_m$  value.

(10) Quantitative, polymorphous analysis method

Determination of the amount of a target gene or the polymorphous composition of the gene is performed with respect to the nucleic acid after amplifying the nucleic acid by the novel quantitative PCR method of the present invention. The amplified nucleic acid is modified with the fluorescent dye. As the fluorescent dye can be analyzed as a marker in the polymorphous analysis, the polymorphous analysis can be conducted easily and quickly with good quantitateness.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 diagrammatically shows changes in the intensity of fluorescence in a solution system with a nucleic acid probe according to the present invention contained therein when a target nucleic acid was added, in which time (sec) is plotted along the abscissa and intensities of fluorescence are plotted along the ordinate;

FIG. 2 shows a working curve for a target nucleic acid by a nucleic acid probe according to the present invention, in

which concentrations of the target nucleic acid are plotted along the abscissa and intensities of fluorescence are plotted along the ordinate;

FIG. 3 illustrates probe designs and target nucleic acid designs for studying effects of the distance (the number of bases) between a fluorescent dye (Texas Red) and a quencher substance (Dabcyl) on the emission of fluorescence from a fluorescence emitting probe making use of interaction between the fluorescent dye and the quencher substance;

FIG. 4 is a diagram illustrating effects of the distance (the number of bases) between the fluorescent dye (Texas Red) and the quencher substance (Dabcyl) on the emission of fluorescence from the fluorescence emitting probe making use of interaction between the fluorescent dye and the quencher substance, in which:

Open column: Fluorescence intensity after hybridization  
(absolute value of fluorescence intensity,  
measuring wave length: 623.5 nm),

Closed column: Fluorescence intensity before  
hybridization (absolute value of  
fluorescence intensity, measuring wave  
length: 623.5 nm), and

—●—: Fluorescence intensity before  
hybridization/fluorescence intensity after  
hybridization;

FIG. 5 illustrates probe designs, in each of which bases in a deoxyribooligonucleotide chain were modified with both fluorescent dye (Texas Red) and quencher substance (Dabcyl), respectively, and target nucleic acid designs;

5        FIG. 6 is a diagram illustrating effects of the distance (the number of bases) between the fluorescent dye (Texas Red) and the quencher substance (Dabcyl) on the emission of fluorescence as observed using a probe in which bases in a deoxyribooligonucleotide chain were modified with both of the fluorescent dye and the quencher substance, respectively;

10        FIG. 7 is a diagram showing measurement data of fluorescence intensity when the sequence of bases in 16S rRNA of *Escherichia coli*, said bases ranging from the 335<sup>th</sup> base to the 358<sup>th</sup> base as counted from the 5' end, was determined using  
15        a nucleic acid probe obtained in Example 7;

FIG. 8 diagrammatically illustrates effects of heat treatment of a target nucleic acid on hybridization of a 35-nucleotides-chained 2-O-Me probe to the target nucleic acid, in which:

20        Dashed curve: rRNA was added as a target nucleic acid subsequent to its heat treatment, and

Solid curve: rRNA not subjected to heat treatment;

FIG. 9 diagrammatically shows effects of the number of bases in a nucleotide chain of a probe, a helper probe and  
25        methylation of an OH group on the 2' carbon of ribose at the 5' end

of the probe on the hybridization between the probe and a target nucleic acid, 16S rRNA, in which:

Ref: Reference

In the references of the probes A,B, 35-base oligonucleotide was used as a target nucleic acid.

In the references of the probes C,D, 17-base oligonucleotide was used as a target nucleic acid;

FIG. 10 shows a working curve for rRNA assay by an invention method;

FIG. 11 diagrammatically shows analysis results of the time-dependent rRNA amount of strains, KYM-7 and KYM-8, in co-cultivation by a FISH method according to the present invention;

FIG. 12 is a schematic illustration of a DNA chip according to the present invention, in which MP-10MH-PG microheaters were used;

FIG. 13 is a schematic illustration of equipment for an SNAPS detection or determination making use of the DNA chip according to the present invention;

FIG. 14 is a diagram showing experimental results of the SNAPS detection or determination making use of the DNA chip according to the present invention;

FIG. 15 diagrammatically illustrates a relationship between cycles and a decrease in fluorescence emission from a fluorescent dye in a quantitative PCR method making use of

primers 1 and 2 labeled with "BODIPY FL/C6", in which signs ① to ⑧ have the following meanings:

- ① Number of copies of *E. coli* genome DNA: 0;  
primer: primer 1 + primer 2.
- ② Number of copies of *E. coli* genome DNA:  $2.4 \times 10^6$ ;  
primer: same as above.
- ③ Number of copies of *E. coli* genome DNA:  $2.4 \times 10^5$ ;  
primer: same as above.
- ④ Number of copies of *E. coli* genome DNA:  $2.4 \times 10^4$ ;  
primer: same as above.
- ⑤ Number of copies of *E. coli* genome DNA:  $2.4 \times 10^3$ ;  
primer: primer 1.
- ⑥ Number of copies of *E. coli* genome DNA:  $2.4 \times 10^2$ ;  
primer: same as above.
- ⑦ Number of copies of *E. coli* genome DNA:  $2.4 \times 10^1$ ;  
primer: same as above.
- ⑧ Number of copies of *E. coli* genome DNA:  $2.4 \times 10^0$ ;  
primer: same as above.

FIG. 16 diagrammatically shows a relationship between cycles and the logarithm of a decrease in fluorescence emission from a fluorescent dye in the quantitative PCR making use of primers 1 and 2 labeled with "BODIPY FL/C6", in which signs ① to ⑧ have the same meanings as defined above in connection with FIG. 15;

FIG. 17 is a diagram showing a working line for 16S rDNA

of *Escherichia coli*, which was prepared using the quantitative PCR according to the present invention;

FIG. 18 (upper diagram) depicts decreases (%) in fluorescence intensity in real-time quantitative PCR according to the present invention in which a single probe of the present invention was used as opposed to two probes labeled with a fluorescent dye and required for a conventional real-time quantitative PCR method using FRET;

FIG. 18 (lower diagram) shows a working line prepared by calculating numbers of cycles (threshold numbers: Ct values) at which decreases in fluorescence intensity were begun to be significantly observed;

FIG. 19 depicts fluorescence decrease curves obtained by real-time quantitative PCR, which used an invention primer labeled with "BODIPY FL/C6", without performing correction processing according to the present invention, in which:

- Target nucleic acid: 10 copies; Temperature of the reaction system upon measurement of fluorescence intensity: 72°C.
- Target nucleic acid: 100 copies; Temperature of the reaction system upon measurement of fluorescence intensity: 72°C.
- ▲ Target nucleic acid: 1,000 copies; Temperature of the reaction system upon measurement of fluorescence intensity: 72°C.

◆ Target nucleic acid: 10,000 copies; Temperature of the reaction system upon measurement of fluorescence intensity: 72°C.

□ Target nucleic acid: 10 copies; Temperature of the reaction system upon measurement of fluorescence intensity: 95°C.

○ Target nucleic acid: 100 copies; Temperature of the reaction system upon measurement of fluorescence intensity: 95°C.

△ Target nucleic acid: 1,000 copies; Temperature of the reaction system upon measurement of fluorescence intensity: 95°C.

◇ Target nucleic acid: 10,000 copies; Temperature of the reaction system upon measurement of fluorescence intensity: 95°C.

FIG. 20 shows fluorescence decrease curves obtained by the real-time quantitative PCR in FIG. 19 except that on each of the curves, each decrease (%) in fluorescence emission was corrected assuming that the corresponding value in the 10<sup>th</sup> cycle was 1, in which:

■ Target nucleic acid: 10 copies; Temperature upon measurement of fluorescence intensity: 72°C.

● Target nucleic acid: 100 copies; Temperature upon measurement of fluorescence intensity: 72°C.

▲ Target nucleic acid: 1,000 copies; Temperature upon

measurement of fluorescence intensity: 72°C.

- ◆ Target nucleic acid: 10,000 copies; Temperature upon measurement of fluorescence intensity: 72°C.

FIG. 21 shows curves obtained by calculating, with respect to the individual plotted values on the respective curves in FIG. 20, the rates of decreases (the rates of changes) in fluorescence intensity in accordance with the formula (9) and then plotting the thus-calculated values, in which:

- Target nucleic acid: 10 copies.
- Target nucleic acid: 100 copies.
- ▲ Target nucleic acid: 1,000 copies.
- ◆ Target nucleic acid: 10,000 copies.

FIG. 22 shows a working line for human genome DNA as obtained from the data in FIG. 21, in which:

- y: Number of copies of human  $\beta$ -globin gene,
- x: cycles (Ct), and
- $R^2$ : correlation coefficient.

FIG. 23 depicts curves obtained by subjecting the measurement values in the individual cycles in FIG. 19 to correction processing in accordance with the formula (1) and then plotting the corrected values relative to their corresponding cycles, in which:

- Target nucleic acid: 10 copies.
- Target nucleic acid: 100 copies.
- ▲ Target nucleic acid: 1,000 copies.

◆ Target nucleic acid: 10,000 copies.

FIG. 24 illustrates curves obtained by plotting values, which had been obtained by processing the processed values of the individual cycles in FIG. 23 in accordance with the formula (3), against their corresponding cycles, in which

■ Target nucleic acid: 10 copies.

● Target nucleic acid: 100 copies.

▲ Target nucleic acid: 1,000 copies.

◆ Target nucleic acid: 10,000 copies.

FIG. 25 shows curves obtained by subjecting the corrected values in the individual cycles in FIG. 24 to correction processing in accordance with the formula (6) and then plotting the corrected values relative to their corresponding cycles, in which:

■ Target nucleic acid: 10 copies.

● Target nucleic acid: 100 copies.

▲ Target nucleic acid: 1,000 copies.

◆ Target nucleic acid: 10,000 copies.

FIG. 26 shows working lines drawn corresponding to 0.1, 0.3, 0.5, 0.7, 0.9 and 1.2 chosen at will as candidates for  $C_t$  values from the respective values of  $\log$  (change in fluorescence, %) in FIG. 24, in which the individual working lines have the following correlation coefficients:

▲  $\log_{10}$  (change in fluorescence, %) = 0.1;

correlation coefficient: 0.998

■  $\log_{10}$  (change in fluorescence, %) = 0.3;

correlation coefficient: 0.999

●  $\log_{10}$  (change in fluorescence, %) = 0.5;

correlation coefficient: 0.9993

△  $\log_{10}$  (change in fluorescence, %) = 0.7

correlation coefficient: 0.9985

□  $\log_{10}$  (change in fluorescence, %) = 0.9

correlation coefficient: 0.9989

○  $\log_{10}$  (change in fluorescence, %) = 1.2

correlation coefficient: 0.9988

FIG. 27 depicts fluorescence decrease curves when real-time quantitative PCR was conducted on human genome DNA of 1 copy and 10 copies by using an invention primer labeled with "BODIPY FL/C6" and the correction processing of the formula

(1) was applied, in which:

1: target nucleic acid = 0 copy,

2: target nucleic acid = 1 copy, and

3: target nucleic acid = 10 copies;

FIG. 28 illustrates melting curves of nucleic acids when a melting curve analysis was conducted with respect to the PCR amplification products shown in FIG. 27, in which:

1: target nucleic acid = 0 copy,

2: target nucleic acid = 1 copy, and

3: target nucleic acid = 10 copies;

FIG. 29 illustrates curves obtained by differentiating

the curves of FIG. 28 and showing  $T_m$  values as valleys, in which:

2: target nucleic acid: 1 copy, and

3: target nucleic acid: 10 copies;

FIG. 30 shows amplification curves of 16S rRNA genes (cDNAs) obtained using quantitative PCT according to the present invention, in which:

Solid curves: cDNA of *Escherichia coli*

Dotted curve: Polymorphous cDNA

$10^2, 10^3, 10^4, 10^5, 10^6$ : Numbers of copies;

FIG. 31 illustrates a working line for cDNA, which was prepared by a data analysis method according to the present invention, in which:

a: 288,000 copies;

FIG. 32 illustrates an analysis pattern by polymorphous T-RELP according to the present invention, in which

bp: Number of base pairs;

FIG. 33 diagrammatically illustrates results of quantitative PCR making use of a fluorescence emitting probe as a primer (fluorescence emitting primer) (exponential graph);

FIG. 34 shows a working line for 16S rRNA gene (fluorescence emitting primer: 0  $\mu$ M), in which:

A: Number of copies in an artificial co-cultivation system of microorganisms (about 296,000 copies);

FIG. 35 (upper diagram) illustrates results of real-time monitoring on PCR amplification products obtained by

real-time quantitative PCR making use of a fluorescence emitting primer;

FIG. 35 (lower diagram) shows a working line obtained by the real-time monitoring;

FIG. 36 diagrammatically shows results of an SNPs detection by a fluorescence emitting probe, in which:

----- : 100% matching target nucleic acid (denaturation curve from a fluorescence emitting probe),

----- : Target nucleic acid containing single nucleotide polymorphism (denaturation curve from a fluorescence emitting probe),

----- : 100% matching target nucleic acid (denaturation curve from a fluorescence quenching probe),

----- : Target nucleic acid containing single nucleotide polymorphism (denaturation curve from a fluorescence quenching probe),

A: Percent fluorescence emission - data of fluorescence emitting probe,

B: Relative value of fluorescence - data of fluorescence quenching probe,

a: About 46°C, and

b: About 46°C;

FIG. 37 diagrammatically illustrates results of an SNPs detection by a DNA chips with fluorescence emitting probes fixed thereon, in which:

—■— : No. 1 100% matching  
 .....□.....: No. 1 1 base mismatched  
 —▲— : No. 2 100% matching  
 .....△..... : No. 2 1 base mismatched  
 —●— : No. 3 100% matching  
 .....○.....: No. 3 1 base mismatched  
 —◆— : No. 4 100% matching  
 .....◇..... : No. 4 1 base mismatched  
 ——— : No. 5 100% matching  
 - - - - - : No. 5 1 base mismatched;

FIG. 38 diagrammatically shows results of real-time monitoring of PCR reaction using a DNA array having fixed fluorescence emitting probes and fluorescence quenching probes, in which:

Closed square: WIAF-10600 (No. 1)  
 Open square: WIAF-10578 (No. 2)  
 Closed circle: WIAF-10600 (No. 3)  
 Open circle: WIAF-10578 (No. 4)  
 Fn: Relative fluorescence rate at n cycle  
 Rn: Fluorescent quenching rate at n cycle;  
 and

FIG. 39 depicts melting curves of PCR products using a DNA array having fixed fluorescence emitting probes and fluorescence quenching probes, in which:

Closed square: WIAF-10600 (No. 1)

Open square: WIAF-10578 (No. 2)

Closed circle: WIAF-10600 (No. 3)

Open circle: WIAF-10578 (No. 4)

Fn, Rn: Same meanings as defined above in  
connection with FIG. 38.

### DETAILED DESCRIPTION OF THE INVENTION

#### AND PREFERRED EMBODIMENTS

The present invention will next be described in further  
detail based on certain preferred embodiments.

The present invention has three aspects.

The present invention, in the first aspect thereof,  
relates to a novel nucleic acid probe for determining a  
concentration of a target nucleic acid, comprising:

a single-stranded oligonucleotide capable of hybridizing  
to the target nucleic acid, and

a fluorescent dye and a quencher substance, both of which  
are labeled on the oligonucleotide,

wherein the oligonucleotide is labeled with the  
fluorescent dye and the quencher substance such that an  
intensity of fluorescence in a hybridization reaction system  
increases when the nucleic acid probe is hybridized with the  
target nucleic acid; and the oligonucleotide forms no stem-  
loop structure between bases at positions where the  
oligonucleotide is labeled with the fluorescent dye and the

quencher substance, respectively. For the sake of brevity, the nucleic acid probe according to the present invention may therefore be called a "fluorescence emitting probe" or a "nucleic acid probe according to the first aspect of the present invention" in the subsequent description.

The present invention, in the second aspect thereof, relates to a nucleic acid probe labeled with a fluorescent dye, which is characterized in that, when the nucleic acid probe hybridizes to a target nucleic acid, emission of fluorescence from the fluorescent dye decreases after the hybridization. It is to be noted that the nucleic acid probe according to the present invention may also be called a "fluorescence quenching probe" or a "nucleic acid probe according to the second aspect of the present invention" for the sake of brevity.

The present invention, in the third aspect thereof, relates to a variety of use of the fluorescence emitting probe and fluorescence quenching probe.

A description will now be made about technical terms employed in the present invention.

The term "probe-nucleic acid hybrid complex" as used herein means one (complex) in which a nucleic acid probe according to the present invention, which is labeled with a fluorescent dye, and a target nucleic acid are hybridized with each other. For the same of brevity, it will be called a "nucleic acid hybrid complex" in a shortened form.

Further, the term "fluorescent dye-nucleic acid conjugate" as used herein means a conjugate in which a fluorescent dye is bound with a target nucleic acid. Illustrative is a conjugate in which an intercalator is bound in a double-stranded nucleic acid.

The terms as used herein - such as to hybridize, hybridization, stem-loop structures, quenching, quenching effects, DNAs, RNAs, cDNAs, mRNAs, rRNAs, XTPs, dXTPs, NTPs, dNTPs, nucleic acid probes, helper nucleic acid probes (or nucleic acid helper probes, or simply helper probes), to hybridize, hybridization, intercalators, primers, annealing, extending reactions, thermal denaturing reactions, nucleic acid melting curves, PCR, RT-PCR, RNA-primed PCR, stretch PCR, reverse PCR, PCR using Alu sequence(s), multiple PCR, PCR using mixed primers, PCR using PNA, hybridization assays, FISH methods (fluorescent in situ hybridization assays), PCR methods (polymerase chain assays), LCR methods (ligase chain reactions), SD methods (strand displacement assays), competitive hybridization, DNA chips, nucleic acid detecting (gene-detecting) devices, SNP (single nucleotide polymorphism), and co-cultivation systems of plural microorganisms - have the same meanings as the corresponding terms generally employed these days in molecular biology, genetic engineering, bioengineering and the like.

The term "target gene" or "target nucleic acid" as used

herein means a gene or a nucleic acid the quantitation or qualitative detection or mere detection of which is intended, irrespective whether it is in a purified form or not and further irrespective of its concentration. Various other nucleic acids may also exist together with the target nucleic acid. For example, the target nucleic acid may be a specific nucleic acid in a co-cultivation system microorganisms (a mixed system of RNAs or gene DNAs of plural microorganisms) or a symbiotic cultivation system of microorganisms (a mixed system of RNAs or gene DNAs of plural animals, plants and/or microorganisms), the quantitation or qualitative detection or mere detection of which is intended. Purification of the specific nucleic acid, if needed, can be conducted by a method known per se in the art. For example, purification can be effected using a purification kit or the like available on the market. Specific examples of the above-described nucleic acid can include DNAs, RNAs, PNAs, oligodeoxyribonucleotides, and oligoriboxynucleotides. Other examples can include chimera nucleic acids of the above-exemplified nucleic acids.

The expression "to determine a concentration of a target nucleic acid" as used herein means to quantitatively determine concentration(s), to perform qualitative detection, to simply detect, or to perform an analysis for polymorphism and/or mutation, all with respect to one or more nucleic acids in a measurement system. In the case of plural nucleic acids,

quantitative detection of the plural nucleic acids at the same time, simple detection of the plural nucleic acids at the same time and an analysis for the polymorphism, mutation and/or the like of the plural nucleic acids at the same time obviously fall within the technical scope of the present invention.

The term "device for the measurement of a concentration of a target nucleic acid" as used herein mean various DNA chips. Specific examples of the device can obviously include a variety of DNA chips. The present invention include all DNA chips irrespective of their types insofar as the nucleic acid probe according to the present invention can be applied to them.

The expression "method for determining a concentration of a target nucleic acid by using a nucleic acid probe labeled with a fluorescent dye (hereinafter simply called a "nucleic acid probe according to the present invention" or a "probe according to the present invention" means to determine the concentration of the target nucleic acid by a hybridization assay, FISH method (fluorescent in situ hybridization assay), PCR method (polymerase chain assay), LCR method (ligase chain reaction), SD method (strand displacement assay), competitive hybridization or the like.

A description will first be made of the fluorescence emitting probes.

This probe is characterized in that, when the probe is not hybridized with a target nucleic acid, emission of

fluorescence from the fluorescent dye is inhibited by the quencher substance but, when the probe is hybridized with the target nucleic acid, the inhibition is rendered ineffective to result in an increase in the intensity of fluorescence.

5       The term "fluorescent dye" as used herein means fluorescent dyes of the like, which are generally used for the determination or detection of nucleic acids by labeling nucleic acid probes. Illustrative of such fluorescent dyes are fluorescein and derivatives thereof [for example, fluorescein isothiocyanate (FITC) and its derivatives]; Alexa 488, Alexa 10       532, cy3, cy5, 6-joe, EDANS; rhodamine 6G (R6G) and its derivatives [for example, tetramethylrhodamine (TMR), tetramethylrhodamine isothiocyanate (TMRITC), x-rhodamine, Texas red, "BODIPY FL" (trade name, product of Molecular Probes, Inc. (Eugene, Oregon, U.S.A.), "BODIPY FL/C3" (trade name, 15       product of Molecular Probes, Inc.), "BODIPY FL/C6" (trade name, product of Molecular Probes, Inc.), "BODIPY 5-FAM" (trade name, product of Molecular Probes, Inc.), "BODIPY TMR" (trade name, product of Molecular Probes, Inc.), and derivatives thereof 20       (for example, "BODIPY TR" (trade name, product of Molecular Probes, Inc.), "BODIPY R6G" (trade name, product of Molecular Probes, Inc.), "BODIPY 564" (trade name, product of Molecular Probes, Inc.), and "BODIPY 581" (trade name, product of Molecular Probes, Inc.) ]. Among these, FITC, EDANS, Texas red, 25       6-joe, TMR, Alexa 488, Alexa 532, "BODIPY FL/C3" and "BODIPY

FL/C6" are preferred, with EDANS, Texas red, FITC, TMR, 6-joe, "BODIPY FL/C3" and "BODIPY FL/C6" being more preferred.

The term "quencher substance" means a substance which acts on the above-described fluorescent dye and inhibits or quenches emission of fluorescence from the fluorescent dye. Illustrative are Dabcyl, "QSY7" (Molecular Probes), "QSY33" (Molecular Probes), Ferrocene and its derivatives, methyl viologen, and N,N'-dimethyl-2,9-diazopyrenium, with Dabcyl and the like being preferred.

By labeling an oligonucleotide at specific positions thereof with such fluorescent dye and quencher substance as described above, the emission of fluorescence from the fluorescent dye is subjected to quenching effect by the quencher substance.

The expression "single-stranded oligonucleotide, which forms a nucleic acid probe according to the present invention and forms no stem-loop structure between bases at positions where the oligonucleotide is labeled with the fluorescent dye and the quencher substance, respectively" means an oligonucleotide which - owing to the complementation of base sequences at at least two positions between the bases at positions where the oligonucleotide is labeled with the fluorescent dye and the quencher substance, respectively - forms double strands in its own chain and forms no stem-loop structure.

To label an oligonucleotide useful in the practice of the present invention with a fluorescent dye and a quencher substance such that the intensity of fluorescence in a hybridization reaction system increases when the resulting nucleic acid probe according to the present invention is hybridized with a target nucleic acid, the labeling can be conducted as will be described hereinafter.

The distance between the bases at the positions where the single-stranded oligonucleotide is labeled with the fluorescent dye and the quencher substance, respectively, is zero (0) in terms of the number of bases, that is, the single-stranded oligonucleotide is labeled at the same position of the same nucleotide thereof with the fluorescent dye and the quencher substance. As an alternative, the distance is 1 to 20 or  $\{(a \text{ desired integer of from } 3 \text{ to } 8) + 10n\}$  ( $n$ : an integer  $\geq 0$ ) in terms of the number of bases. More preferably, the single-stranded oligonucleotide can be labeled at the same position of the same nucleotide thereof or can be labeled with a distance of from 4 to 8. It is desired to label an oligonucleotide with a fluorescent dye and a quencher substance, respectively, as described above. However, the distance between the bases depends strongly upon the base sequence of the probe, the fluorescent dye and quencher substance to be used for modification, the lengths of linkers adapted to bind them to the oligonucleotide, and the like. It is, therefore,

difficult to fully specify the base-to-base distance. It is to be noted that the above-described base-to-base distances are merely general examples and the distance between the bases includes many exceptions.

Concerning the labeling positions, it is preferred that, when a single-stranded oligonucleotide is labeled at the position of the same nucleotide thereof, one of a fluorescent dye and a quencher substance is labeled to a base and the other is labeled to a portion other than bases, specifically to a phosphate portion or to a ribose portion or deoxyribose portion. In this case, labeling to the 3' end portion or 5' end portion is preferred.

When it is desired to set the distance between the bases labeled with the fluorescent dye and quencher substance, respectively, at 1 to 20 or  $\{(a \text{ desired integer of from } 3 \text{ to } 8) + 10n\}$  ( $n$ : an integer  $\geq 0$ ), preferably at 4 to 8 or a number obtained by adding 10 to a desired number in this range, more preferably at 4 to 8 in terms of the number of bases, the oligonucleotide may be labeled in its chain with both of the fluorescent dye and quencher substance or may be labeled at the 5' end or 3' end thereof with one of the fluorescent dye and quencher substance and in the chain thereof with the other one. It is preferred to label the oligonucleotide at the 5' end or 3' end thereof with the fluorescent dye or the quencher substance and at a base, which is apart by the above-described number of

bases from the end, with the quencher substance or the fluorescent dye. When it is desired to label the 3' end or 5' end in this case, the labeling can be effected to a base, a phosphate portion, a ribose portion or a deoxyribose portion, preferably to the phosphate portion, the ribose portion or the deoxyribose portion, more preferably to the phosphate portion. When it is desired to conduct the labeling into the chain, the labeling can be effected preferably to bases in the chain.

When the bases are modified in each of the above-described cases, the modification can be effected to any bases insofar as the modification is feasible. It is, however, preferred to effect the modification to the OH group, amino group, 2-N, 7-N and/or 8-C of a purine base or to the OH group, amino group, methyl group and/or 2-N of a pyrimidine base [ANALYTICAL BIOCHEMISTRY, 225, 32-38 (1998)].

The nucleic acid probe according to the present invention, which is to be hybridized to the target nucleic acid, may be formed of either an oligodeoxyribonucleotide or an oligoribonucleotide. The nucleic acid probe may be a chimeric oligonucleotide which contains both of them. These oligonucleotides may be in chemically-modified forms. Such chemically-modified oligonucleotides may be inserted in chimeric oligodeoxynucleotides.

Examples of the modified positions of the chemically-modified oligonucleotide can include an end hydroxyl group or

end phosphate group of an end portion of an oligonucleotide, the position of a phosphate portion of an internucleoside, the 5-carbon of a pyrimidine ring, and the position of a saccharide (ribose or deoxyribose) in a nucleoside. Preferred examples are the positions of ribose or deoxyribose. Specific examples can include 2'-O-alkyloligoribonucleotides ("2'-O-" will hereinafter be abbreviated as "2-O-"), 2-O-alkyleneoligoribonucleotides, and 2-O-benzyloligoribonucleotides. The oligonucleotide is modified at the OH group(s) on the 2' carbon(s) of one or more ribose molecules at desired positions thereof with alkyl group(s), alkylene group(s) or benzyl group(s) (via ether bond(s)). Preferred examples useful in the present invention can include, among 2-O-alkyloligoribonucleotides, 2-O-methyloligoribonucleotide, 2-O-ethyloligoribonucleotide and 2-O-butyloligoribonucleotide; among 2-O-alkyleneoligoribonucleotides, 2-O-ethyleneoligoribonucleotide; and 2-O-benzyloligoribonucleotide. Particularly preferably, 2-O-methyloligoribonucleotide (hereinafter simply abbreviated as "2-O-Me-oligoribonucleotide") can be used. Application of such chemical modification to an oligonucleotide enhances its affinity with a target nucleic acid so that the efficiency of hybridization with a nucleic acid probe according to the present invention is improved. The improved efficiency of hybridization leads to a further improvement in the rate of a decrease in the

intensity of fluorescence from the fluorescent dye of the nucleic acid probe according to the present invention. As a consequence, the accuracy of determination of the concentration of the target nucleic acid is improved further.

5           Incidentally, it is to be noted that the term "oligonucleotide" as used herein means an oligodeoxyribonucleotide or an oligoribonucleotide or both of them and hence, is a generic term for them.

10           2-O-alkyloligoribonucleotides, 2-O-alkyleneoligoribonucleotides and 2-O-benzyloligoribonucleotide can be synthesized by a known process [Nucleic Acids Research, 26, 2224-2229 (1998)]. As custom DNA synthesis services are available from GENSET SA, Paris, France, they can be readily obtained. The present inventors have completed the present  
15           invention by conducting experiments with the compounds furnished by this company pursuant to our order.

20           Incidentally, use of a nucleic acid probe according to the present invention with modified DNA, such as 2-O-methyloligoribonucleotide (hereinafter simply called "2-O-Me-oligoribonucleotide), inserted in an oligodeoxyribonucleotide primarily for the determination of RNA, especially for the determination of rRNA can provide preferred results.

25           Upon determination of RNA by the nucleic acid probe according to the present invention, it is preferred to subject

an RNA solution as a sample to heat treatment at 80 to 100°C, preferably 90 to 100°C, most preferably 93 to 97°C for 1 to 15 minutes, preferably 2 to 10 minutes, most preferably 3 to 7 minutes before hybridization with the probe such that the higher-order structure of RNA can be degraded, as this heat treatment makes it possible to improve the efficiency of hybridization.

It is also preferred to add a helper probe to a hybridization reaction mixture for raising the efficiency of hybridization of the nucleic acid probe of this invention to the hybridization sequence region. In this case, the oligonucleotide of the helper probe can be in an oligodeoxyribonucleotide, an oligoribonucleotide or an oligonucleotide subjected to similar chemical modification as described above. Examples of the above-described oligonucleotides can include those having the base sequence of (5')TCCTTTGAGT TCCCGGCCGG A(3') as the forward type and those having the base sequence of (5')CCCTGGTCGT AAGGGCCATG ATGACTTGAC GT(3') as the backward type or the reverse type. Preferred examples of the chemically-modified oligonucleotide can include 2-O-alkyloligoribonucleotides, notably 2-O-Me-oligoribonucleotide.

Where the base strand of the nucleic acid probe according to the present invention is formed of 35 or fewer bases, use of a helper probe is particularly preferred. When a nucleic

acid probe according to the present invention longer than a 35-base strand is used, however, it may only be necessary to thermally denature target RNA in some instances.

When the nucleic acid probe according to the present invention is hybridized to RNA as described above, the efficiency of hybridization is enhanced. The fluorescence intensity, therefore, decreases corresponding to the concentration of RNA in the reaction mixture so that RNA can be determined up to a final RNA concentration of about 150 pM.

Accordingly, the present invention also relates to a kit for determining a concentration of a target nucleic acid, which includes or is accompanied by the above-described helper probe in addition to a kit which is adapted to determine the concentration of the target nucleic acid the nucleic acid probe of the present invention and which includes or is accompanied by the nucleic acid probe of this invention.

In determination of RNA by a conventional hybridization assay making use of a nucleic acid probe, an oligodeoxy-ribonucleotide or oligoribonucleotide has been used as the nucleic acid probe. Because RNA itself has a higher-order solid structure, the efficiency of hybridization between the probe and the target RNA was poor, resulting in quantitation of low accuracy. The conventional methods, therefore, are accompanied by irksomeness that a hybridization reaction is conducted after denaturing RNA and immobilizing denatured RNA

on a membrane. The method according to the present invention, on the other hand, uses a nucleic acid probe a ribose portion of which has been modified to have high affinity to a particular structural part of RNA, so that a hybridization reaction can be conducted at a higher temperature compared with the conventional methods. The above-mentioned adverse effects of the high-order structure of RNA can be overcome by simply conducting thermal denaturation as pretreatment and using a helper probe in combination. As a consequence, the efficiency of hybridization in the method according to the present invention is practically as high as 100%, leading to improvements in the accuracy of quantitation. Further, the method according to the present invention is far simpler and easier than the conventional methods.

The probe according to the present invention is formed of 5 to 50 bases, preferably 10 to 25 bases, most preferably 15 to 20 bases. A base number greater than 50 leads to lower permeability through a cell membrane when employed in the FISH method, thereby narrowing an applicable range of the present invention. A base number smaller than 5, on the other hand, tends to induce non-specific hybridization and, therefore, results in a large determination error.

The oligonucleotide in the nucleic acid probe in the present invention can be produced by a conventional production process for general oligonucleotides. It can be produced, for

example, by a chemical synthesis process or by a microbial process which makes use of a plasmid vector, a phage vector or the like (Tetrahedron Letters, 22, 1859-1862, 1981; Nucleic Acids Research, 14, 6227-6245, 1986). Further, it is suitable to use a nucleic acid synthesizer currently available on the market (for example, "ABI394", trade name, manufactured by Perkin-Elmer Corp., Norwalk, Connecticut, U.S.A.). Further, there are some enterprises which offer custom DNA synthesis services on commercial basis. It is, therefore, most convenient to conduct only the designing of base sequences and to order their synthesis to such enterprises. Illustrative of such enterprises are Takara Shuzo Co., Ltd., Kyoto, Japan and Espec Oligo Service Corp., Ibaraki, Japan.

To label the oligonucleotide with the fluorescent dye and the quencher substance, desired one of conventionally-known labeling methods can be used (Nature Biotechnology, 14, 303-308, 1996; Applied and Environmental Microbiology, 63, 1143-1147, 1997; Nucleic Acids Research, 24, 4532-4535, 1996). To conjugate a fluorescent dye and a quencher substance to the 5' end, a linker or spacer, for example,  $-(CH_2)_n-SH$  or  $-(CH_2)_n-NH_2$  is first introduced into a phosphate group at the 5' end by a method known per se in the art. As such a linker- or spacer-introduced derivative is available on the market, a commercial product may be purchased (Midland Certified Reagent Company). In the above-mentioned example, n ranges from 3 to

8 with 6 or 7 being preferred. The oligonucleotide can be labeled by reacting a SH- or NH<sub>2</sub>- reactive fluorescent dye or a quencher substance to the linker or spacer. Reversed phase chromatography or the like to provide a nucleic acid probe for use in the present invention can purify the thus-synthesized oligonucleotide, which is labeled with the fluorescent dye.

Further, to conjugate the fluorescent dye or quencher substance to the 3' end of the oligonucleotide, a linker, for example,  $-(CH_2)_n-NH_2$  or  $-(CH_2)_n-SH$  is introduced onto an OH group on the C atom at the 2'-position or 3'-position of ribose or onto an OH group on the C atom at the 3'-position of deoxyribose. As such a linker-introduced derivative is also available on the market like the above-described ones, a commercial product may be purchased (Midland Certified Reagent Company). As an alternative, a phosphate group may be introduced onto the OH group, followed by the introduction of a linker, for example,  $-(CH_2)_n-SH$  or  $-(CH_2)_n-NH_2$  onto the OH group of the phosphate group. In these cases, n ranges from 3 to 9, with 4 to 8 being preferred. The oligonucleotide can be labeled by reacting an NH<sub>2</sub>- or SH-reactive fluorescent dye or a quencher substance to the linker.

For the introduction of the amino group, it is convenient to use a kit reagent [for example, "Uni-link Aminomodifier" (trade name, product of Clontech Laboratories, Inc., Palo Alto, California, U.S.A.), or "FluoReporter Kit F-6082, F-6083,

F-6084 or F-10220" (trade name, product of Molecular Probes, Inc.)). In a manner known per se in the art, molecules of the fluorescent dye can then be conjugated to the oligoribonucleotide. It is also possible to introduce molecules of the fluorescent dye into strands of the probe nucleic acid (ANALYTICAL BIOCHEMISTRY, 225, 32-38, 1998).

When it is desired to introduce an amino group onto a ribose portion, deoxyribose portion, phosphate portion or base portion of an oligonucleotide, a linker, a fluorescent dye or a quencher substance to enhance its reactivity, use of a kit reagent (for example, "Uni-link Aminomodifier", "FluoReporter Kit F-6082, F-6083, F-6084 or F-10220" is convenient. The fluorescent dye and the quencher substance can then be bound to the oligoribonucleotide by a method known per se in the art.

In the above-described synthesis, the introduction of a protecting group to each function group and the elimination of the protecting group can be conducted by conventional, known methods.

The oligonucleotide labeled with the fluorescent dye and the quencher substance can be synthesized as described above. It is desired to purify intermediate synthesis products and the completed synthesis product by liquid chromatography such as reversed phase liquid chromatography. The nucleic acid probe according to the present invention can be obtained as described above.

As is appreciated from the foregoing, the nucleic acid probe according to the present invention can be designed by simply labeling an oligonucleotide, which has a base sequence hybridizable to a target nucleic acid, with a fluorescent dye and a quencher substance. Its designing is therefore simple.

The nucleic acid probe according to the present invention can be readily can also be readily obtained by ordering its synthesis like the synthesis of the oligonucleotide, provided that only the designing of the probe can be completed.

A description will next be made about the fluorescence quenching probe according to the second aspect of the present invention.

The oligonucleotide of the fluorescence quenching probe of this invention, which is hybridized to a nucleic acid, is similar to that in the above-described fluorescence emitting probe. Specifically, it can be a chimeric oligonucleotide or a chemically-modified oligonucleotide. As a still further alternative, an oligonucleotide with such a chimeric oligonucleotide or chemically-modified oligonucleotide inserted in its chain can also be used.

The position of the oligonucleotide, where the oligonucleotide is modified by a fluorescent dye, is the same as that of the above-described fluorescence emitting probe.

Similarly to the above-described invention, it is also possible to add a helper probe to a hybridization reaction

mixture to further improve the efficiency of hybridization of the nucleic acid probe of this invention to the hybridization sequence region. Further, the base sequence and the number of base chains of the helper probe, the usability of a chemically-modified oligonucleotide, and the like are also as described above in connection with the above-described invention. When hybridized to RNA, the efficiency of hybridization is increased. The intensity of fluorescence is thus decreased corresponding to the amount of RNA in the reaction mixture, thereby making it possible to determine RNA up to a final concentration of about 150 pM.

The thermal modification of RNA and the addition of the helper probe in the determination method of RNA by using the fluorescence quenching probe of this invention are also similar to those described above in connection with the fluorescence emitting probe.

As a consequence, the efficiency of hybridization also reaches substantially 100% in this invention method, leading to an improvement in quantitateness. In addition, the method is far simpler than the conventional methods.

The number of bases in the probe according to the present invention is similar to that in the above-described invention. No particular limitation is imposed on the base sequence of the probe insofar as it specifically hybridizes to the target nucleic acid. Preferred examples of the base sequence of the

probe can include:

(1) a base sequence designed such that at least one G (guanine) base exists in the base sequence of the target nucleic acid at a position 1 to 3 bases apart from the end base portion of the target nucleic acid hybridized to the probe,

(2) a base sequence designed such that plural base pairs of a nucleic acid hybrid complex forms at least one G (guanine) and C (cytosine) pair at an end portion of the probe, and

(3) a base sequence designed such that in the probe modified with the fluorescent label at a portion other than the 5' end phosphate group or the 3' end OH group, base pairs in the fluorescence-labeled portion forms at least one G (guanine) and C (cytosine) pair,

when the nucleic acid probe labeled with the fluorescent dye is hybridized with the target nucleic acid.

The preparation process of the oligonucleotide of the nucleic acid probe according to the present invention and the labeling method of the oligonucleotide with the fluorescent dye are similar to those described above in connection with the above-described invention.

Further, fluorescent dye molecules can also be introduced into the chain of the nucleic acid probe [ANALYTICAL BIOCHEMISTRY, 225, 32-38 (1998)].

The nucleic acid probe according to the present invention can be prepared as described above. A preferred probe form is

one labeled with a fluorescent dye at the 3' or 5' end and containing G or C as the base at the labeled end. If the 5' end is labeled and the 3' end is not labeled, the OH group on the C atom at the 3'-position of the 3' end ribose or deoxyribose or the OH group on the C atom at the 2'-position of the 3' end ribose may be modified with a phosphate group or the like although no limitation is imposed in this respect.

In addition, a nucleic acid probe according to the present invention can also be prepared by modifying C or G in the chain of a probe.

The present invention, in the third aspect thereof, is an invention making use of such fluorescence emitting probes and fluorescence quenching probes as described above.

#### 1) Determination kits and determination devices

A target nucleic acid can be easily and accurately determined in a short time when a fluorescence emitting probe or a fluorescence quenching probe (hereinafter collectively called a "nucleic acid probe of the present invention" for the sake of brevity unless otherwise specifically indicated) is hybridized with the target nucleic acid and a change in the intensity of fluorescence after the hybridization (an increase in the intensity of fluorescence in the case of the fluorescence emitting probes; a decrease in the intensity of fluorescence in the case of the fluorescence quenching probes) is measured. Use of the nucleic acid probe of the present invention also makes

it possible to determine RNA although its determination has heretofore been difficult.

Accordingly, the present invention also relates to a kit for measuring a concentration of a target nucleic acid, which includes or is accompanied by the nucleic acid probe according to the present invention.

Use of the nucleic acid probe according to the present invention is not limited to the determination of a nucleic acid, but it can also be suitably applied to methods for analyzing or determining polymorphism or mutation of a target nucleic acid. In particular, its application to a device for the determination of a concentration of a target nucleic acid {a DNA chip [Tanpakushitsu, Kakusan, Koso (Proteins, Nucleic Acids, Enzymes), 43, 2004-2011 (1998)]} provides a more convenient device for the determination of the concentration of the target nucleic acid. The method for analyzing or determining polymorphism and/or mutation of the target nucleic acid by using the device is an extremely convenient method. Described specifically, when the nucleic acid probe of this invention is a fluorescence quenching probe, the intensity of fluorescence upon its hybridization with the target nucleic acid varies depending on whether or not a GC pair is formed. It is, therefore, possible to analyze or determine polymorphism and/or mutation of a target nucleic acid by hybridizing the nucleic acid probe according to the present invention to the target

nucleic acid and then measuring the intensity of fluorescence. Specific methods will be described in Examples. In this case, the target nucleic acid can be an amplified or extracted product obtained by desired one of nucleic acid amplification methods. Further, no particular limitation is imposed on the kind of the target nucleic acid. They are however required to contain a guanine base or cytosine base in strands thereof or at ends thereof, because the intensity of fluorescence would otherwise not decrease. The method of the present invention can, therefore, analyze or determine a mutation or substitution such as  $G \rightarrow A$ ,  $G \leftarrow A$ ,  $C \rightarrow T$ ,  $C \leftarrow T$ ,  $G \rightarrow C$  or  $G \leftarrow C$ , specifically, polymorphism such as single nucleotide polymorphism (SNP). Incidentally, it is the current practice to perform an analysis of polymorphism by determining the base sequence of a target nucleic acid in accordance with the Maxam-Gilbert method or the dideoxy method.

Inclusion of the nucleic acid probe according to the present invention in a kit for analyzing or determining polymorphism and/or mutation of a target nucleic acid, therefore, makes it possible to suitably use the kit as a kit for the analysis or determination of the polymorphism and/or mutation of the target nucleic acid.

When analyzing data obtained by the method of the present invention for the analysis or determination of polymorphism and/or mutation of a target nucleic acid, a processing step may

be added to correct the intensity of fluorescence, which is emitted from the reaction system when the target nucleic acid is hybridized with the nucleic acid probe of the present invention by the intensity of fluorescence emitted from the reaction system when the target nucleic acid and the nucleic acid probe are not hybridized with each other. The data so processed are provided with high reliability.

Accordingly, the present invention also provides a data analysis method for the method which analyzes or measures polymorphism and/or mutation of a target nucleic acid.

The present invention also features a system for analyzing or determining polymorphism and/or mutation of a target nucleic acid, which has processing means for correcting a fluorescence intensity of a reaction system, in which the target nucleic acid is hybridized with the nucleic acid probe according to the present invention, in accordance with a fluorescence intensity of the reaction system in which the target nucleic acid is not hybridized with the nucleic acid probe according to the present invention.

The present invention further features a computer-readable recording medium with procedures recorded as a program therein for making a computer perform a processing step in which, when analyzing data obtained by the method for analyzing or determining polymorphism and/or mutation of a target nucleic acid, a fluorescence intensity of a reaction system, in which

the target nucleic acid is hybridized with the nucleic acid probe according to the present invention, is corrected in accordance with a fluorescence intensity of the reaction system in which the target nucleic acid or gene is not hybridized with the nucleic acid probe according to the present invention.

The probe according to the present invention may be immobilized on a surface of a solid (support layer), for example, on a surface of a slide glass. In this case, the probe may preferably be immobilized on the end not labeled with the fluorescent dye. The probe of this form is now called a "DNA chip". These DNA chips can be used for monitoring gene expressions, determining base sequences, analyzing mutations or analyzing polymorphisms such as single nucleotide polymorphism (SNP). Needless to day, they can also be used as devices (chips) for determining nucleic acids.

To bind the probe of the present invention, for example, to a surface of a slide glass, a slide glass coated with polycations such as polylysine, polyethyleneimine or polyalkylamine, a slide glass with aldehyde groups introduced thereon, or a slide glass with amino groups introduced thereon is first provided. Binding can then be achieved, for example, by i) reacting phosphate groups of the probe to the slide glass coated with the polycations, ii) reacting a probe, in which amino groups have been introduced, to the slide glass on which aldehyde groups have been introduced or iii) reacting a probe,

in which PDC (pyridinium dichlomite) residual groups, amino groups or aldehyde groups have been introduced, to the slide glass on which amino groups have been introduced (Fodor, P.A., et al., Science, **251**, 767-773, 1991; Schena, W., et al., Proc. Natl. Acad. Sci., U.S.A., **93**, 10614-10619, 1996; McGal, G., et al., Proc. Natl. Acad. Sci., U.S.A., **93**, 13555-13560, 1996; Blanchad, A.P., et al., Biosens. Bioelectron., **11**, 687-690, 1996).

A device having nucleic acid probes of the invention arranged and bound in an arrayed form on a surface of a solid support permits more convenient determination of a nucleic acid.

In this case, the formation of a device by individually binding many probes of this invention, the base sequences of which are different from each other, on a surface of the same solid support makes it possible to simultaneously detect and quantitate a variety of target nucleic acids.

Preferably, this device may be designed such that each probe is provided on a side of the solid support, said side being opposite to the side to which the probe is bound, with at least one temperature sensor and at least one heater at an area of the solid support, where the probe is bound, can be controlled to meet optimal temperature conditions.

For this device, probes other than nucleic acid probes of the present invention, for example, nucleic acid probes of

a construction designed such that two different fluorescent dyes are contained per molecule and each of the probes either quenches or emits fluorescence owing to interaction between the two fluorescent dyes when the probe is not hybridized with its corresponding target nucleic acid but either emits fluorescence or quenches when the probe hybridizes to the target nucleic acid, specifically, a device with molecular beacons described above (Tyagi et al., Nature Biotech., 14, 303-308, 1996) or the like bound thereon can also be used suitably. These devices, therefore, are embraced within the technical scope of the present invention.

Fundamental operations in the determination method making use of the device according to the present invention are simply to place a solution, which contains a target nucleic acid such as mRNA, cDNA or rRNA, on the solid support on which the nucleic probes are bound and then to induce hybridization. As a result, a change in the intensity of fluorescence takes place corresponding to the concentration of the target nucleic acid, and the target nucleic acid can then be detected and quantitated from the change in the intensity of fluorescence. Further, binding of many nucleic acid probes of different base sequences on a surface of a single support makes it possible to determine concentrations of many target nucleic acids at the same time. As this device can be used for exactly the same application as a DNA chip, that is, for the determination of the concentrations

of the target nucleic acids, it is a novel DNA chip. Under reaction conditions optimal for the target nucleic acid, the intensities of fluorescence emitted from the nucleic acids other than the target nucleic acid remain unchanged. No operation is, therefore, needed for washing off the unreacted nucleic acids. Further, independent temperature control of the individual nucleic acid probes according to the present invention by their corresponding microheaters makes it possible to control the probes under their optimal reaction conditions, respectively. Accurate determination of concentrations is therefore feasible. In addition, a denaturation curve between each nucleic acid probe of this invention and its corresponding target nucleic acid can be analyzed by continuously changing the temperature with the microheater and measuring the intensity of fluorescence during the changing of the temperature. From differences in such denaturation curves, it is possible to determine properties of the hybridized nucleic acid and also to detect SNP.

Further, the device also makes it possible to conduct amplification of a gene by PCR or the like and detection of the gene at the same time.

According to each conventional device for determining a concentration of a target nucleic acid, a nucleic acid probe not modified with a fluorescent dye is bound or fixed on a surface of a solid support and, subsequent to hybridization with the

target nucleic acid labeled with the fluorescent dye, an unhybridized portion of the target nucleic acid is washed off, followed by the measurement of the intensity of fluorescence from the remaining fluorescent dye.

5           To label the target nucleic acid with the fluorescent dye, the following steps can be followed, for example, when specific mRNA is chosen as a target: (1) mRNA extracted from cells is extracted in its entirety, and (2) using a reverse transcriptase, cDNA is synthesized while inserting a nucleoside modified by  
10 the fluorescent dye. These operations are not needed in the present invention.

          A number of various probes are applied in spots on the device. Optimal hybridization conditions, for example, temperatures or the like for nucleic acids to be hybridized to  
15 the individual probes are different from each other.

Theoretically speaking, it is therefore necessary to conduct a hybridization reaction and a washing operation under optimal conditions for each probe (at each spot). This is however physically impossible. For all the probes, hybridization is  
20 conducted at the same temperature and further, washing is also carried out at the same temperature with the same washing solution. The device is, therefore, accompanied by a drawback that a nucleic acid does not hybridize although its  
25 hybridization is desired or that, even if its hybridization takes place, the nucleic acid is readily washed off as the

hybridization is not strong. For these reasons, the accuracy of quantitation of the nucleic acid is low. The present invention does not have such a drawback because the above-mentioned washing operation is not needed. Further, a hybridization reaction can be conducted at an optimal temperature for each probe of the present invention by independently arranging a microheater at the bottom of each spot and controlling the hybridization temperature. Accordingly, the accuracy of quantitation has been significantly improved in the present invention.

## 2) Determination method of a target nucleic acid

In the present invention, use of the above-described nucleic acid probe, determination kit or device makes it possible to specifically determine a concentration of a target nucleic acid with ease in a short time. A description will hereinafter be made of the determination method.

In the determination method according to the present invention, the above-described nucleic acid probe is added to a measurement system and is caused to hybridize to a target nucleic acid. This hybridization can be effected by a conventionally-known method (Analytical Biochemistry, 183, 231-244, 1989; Nature Biotechnology, 14, 303-308, 1996; Applied and Environmental Microbiology, 63, 1143-1147, 1997). As conditions for hybridization, the salt concentration may range from 0 to 2 molar concentration, preferably from 0.1 to 1.0 molar

concentration, and the pH may range from 6 to 8, preferably from 6.5 to 7.5.

The reaction temperature may preferably be in a range of the  $T_m$  value of the nucleic acid hybrid complex, which is to be formed by hybridization of the nucleic acid probe to the specific site of the target nucleic acid,  $\pm 10^\circ\text{C}$ . This temperature range can prevent non-specific hybridization. A reaction temperature lower than  $T_m - 10^\circ\text{C}$  allows non-specific hybridization, while a reaction temperature higher than  $T_m + 10^\circ\text{C}$  allows no hybridization. Incidentally, a  $T_m$  value can be determined in a similar manner as in an experiment which is needed to design the nucleic acid probe for use in the present invention. Described specifically, an oligonucleotide which is to be hybridized with the nucleic acid probe of this invention (and has a complementary base sequence to the nucleic acid probe) is chemically synthesized by the above-described nucleic acid synthesizer or the like, and the  $T_m$  value of a nucleic acid hybrid complex between the oligonucleotide and the nucleic acid probe is then measured by a conventional method.

The reaction time may range from 1 second to 180 minutes, preferably from 5 seconds to 90 minutes. If the reaction time is shorter than 1 second, a substantial portion of the nucleic acid probe according to the present invention remains unreacted in the hybridization. On the other hand, no particular advantage can be brought about even if the reaction time is set

excessively long. The reaction time varies considerably depending on the kind of the nucleic acid, namely, the length or base sequence of the nucleic acid.

In the present invention, the nucleic acid probe is hybridized to the target nucleic acid as described above. The intensity of fluorescence emitted from the fluorescent dye is measured both before and after the hybridization, and a decrease in fluorescence intensity after the hybridization is then calculated. As the decrease is proportional to the concentration of the target nucleic acid, the concentration of the target nucleic acid can be determined.

The concentration of the target nucleic acid in the reaction mixture may range from 0.1 to 10.0 nM, while the concentration of the probe in the reaction mixture may range from 1.0 to 25.0 nM. Upon preparation of a working curve, the nucleic acid probe of the present invention may desirably be used at ratios of from 1.0 to 2.5 relative to the target nucleic acid.

Upon actually determining the concentration of a target nucleic acid, the concentration of which is unknown, in a sample, a working curve is first prepared under the below-described conditions. A corresponding probe according to the present invention is added at plural concentrations to aliquots of the sample, respectively, followed by the measurement of changes in the intensity of fluorescence from the respective aliquots.

The probe concentration, which corresponds to the greatest one of the change in fluorescence intensity so measured, is chosen as a preferred probe concentration. Based on the change in fluorescence intensity measured at the preferred probe concentration, a quantitated value of the target nucleic acid can be determined from the working curve.

A description has been made about the principle of the method of the present invention for the determination of a concentration of a nucleic acid. The present invention can be applied to various nucleic acid determination methods, for example, FISH methods, PCR methods, LCR methods, SD methods, competitive hybridizations, and TAS methods.

Examples of these applications will hereinafter be described.

#### ① Application to FISH methods

The method of the present invention can be applied to nucleic acids contained in cells of microorganisms, plants or animals or those contained in homogenates of the respective cells. The method of the present invention can also be suitably applied to nucleic acids in cells of a cultivation system of microorganisms (e.g., a co-cultivation system of microorganisms or a symbiotic cultivation system of microorganisms), in which various kinds of microorganisms are contained together or a microorganism and other animal- or plant-derived cells are contained together and cannot be

isolated from each other, or in a homogenate or the like of the cells of the cultivation system. The term "microorganisms" as used herein means microorganisms in general sense, and no particular limitation is imposed thereon. Examples of such microorganisms can include eukaryotic microorganisms and prokaryotic microorganisms, and also mycoplasmas, virus and rickettsias. The term "a nucleic acid" as used in connection with such a microorganism system means a nucleic acid with a base sequence specific to cells of a cell strain which is desired to be investigated, for example, as to how it is acting in the microorganism strain. Illustrative examples can include 5S rRNAs, 16S rRNAs and 23S rRNAs of certain specific cell strains and particular sequences of their gene DNAs.

According to the present invention, a nucleic acid probe is added to a co-cultivation system of microorganisms or a symbiotic cultivation system of microorganisms and the concentration of 5S rRNA, 16S rRNA or 23S rRNA of a particular cell strain or its gene DNA, thereby making it possible to determine the viable count of the particular strain in the system. Incidentally, a viable count of a particular cell strain in a co-cultivation system of microorganisms or a symbiotic cultivation system of microorganisms can be determined by adding the nucleic acid probe to a homogenate of the system and then measuring a change in fluorescence emission from the fluorescent dye before hybridization relative to fluorescence

emission from the fluorescent dye after the hybridization. It is to be noted that this method also falls within the technical scope of the present invention.

The above-described determination method can be carried out as will be described hereinafter. Before the addition of the nucleic acid probe of the present invention, the temperature, salt concentration and pH of the co-cultivation system of microorganisms or the symbiotic cultivation system of microorganisms are adjusted to meet the conditions described above. It is also preferable to adjust the concentration of the specific cell strain, which is contained in the co-cultivation system of microorganisms or the symbiotic cultivation system of microorganisms, to  $10^7$  to  $10^{12}$  cells/mL, preferably  $10^9$  to  $10^{10}$  cells/mL in terms of viable count. These adjustments can be achieved by dilution, centrifugal or like concentration, or the like. A viable count smaller than  $10^7$  cells/mL results in low fluorescence intensity and greater determination error. A viable count greater than  $10^{12}$  cells/mL, on the other hand, leads to excessively high fluorescence intensity, so that the viable count of the particular microorganism cannot be determined quantitatively. However, this range depends upon the performance of a fluorimeter to be used.

The concentration of the nucleic acid probe of the present invention to be added depends upon the viable count of the

particular cell strain in the co-cultivation system of microorganisms or the symbiotic cultivation system of microorganisms and, at a viable count of  $10^8$  cells/mL, may be in a range of from 0.1 to 10.0 nM, preferably in a range of from 0.5 to 5 nM, more preferably 1.0 nM. A probe concentration lower than 0.1 nM cannot provide any data which accurately reflects the viable count of the particular microorganism. The optimal probe concentration, however, cannot be specified in any wholesale manner because it depends upon the concentration of a target nucleic acid in cells.

Upon hybridizing the nucleic acid probe to the 5S rRNA, 16S rRNA or 23S rRNA of the particular cell strain or its gene DNA in the present invention, the reaction temperature may be set as described above. Further, the hybridization time may also be set as described above.

The nucleic acid probe is hybridized to the 5S rRNA, 16S rRNA or 23S rRNA of the particular cell strain or its gene DNA under such conditions as described above. Intensities of fluorescence from the fluorescent dye in the co-cultivation system of microorganisms or the symbiotic cultivation system of microorganisms before and after the hybridization are then measured.

In the present invention, no particular limitation is imposed on components other than the microorganisms in the co-cultivation system of microorganisms or the symbiotic

cultivation system of microorganisms, insofar as the components do not interfere with the hybridization between the nucleic acid probe according to the present invention and the 5S rRNA, 16S rRNA or 23S rRNA or its gene DNA and further, do not inhibit the emission of fluorescence from the fluorescent dye or the action of the quencher substance labeled on the oligonucleotide. For example, phosphates such as  $\text{KH}_2\text{PO}_4$ ,  $\text{K}_2\text{HPO}_4$ ,  $\text{NaH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$ , inorganic nitrogen compounds such as ammonium sulfate, ammonium nitrate and urea, various salts of metal ions such as magnesium, sodium, potassium and calcium ions, various salts such as the sulfates, hydrochlorides, carbonates and the like of trace metal ions such as manganese, zinc, iron and cobalt ions, and vitamins may be contained to adequate extent. If the above-described interference or inhibition is observed, it may be necessary to separate cells of the plural microorganisms from the cultivation system by an operation such as centrifugal separation and then to resuspend them in a buffer or the like.

Usable examples of the buffer can include various buffers such as phosphate buffer, carbonate buffer, Tris-HCl buffer, Tris-glycine buffer, citrate buffer, and Good's buffer. The buffer should be adjusted to a concentration not inhibiting the hybridization or the emission of fluorescence from the fluorescent dye. This concentration depends upon the kind of the buffer. The pH of the buffer may range from 4 to 12, with 5 to 9 being preferred.

## ② Application to PCR methods

The present invention can be applied to any method insofar as it is a PCR method. A description will hereinafter be made of an application of the present invention to a real-time quantitative PCR method.

In the real-time quantitative PCR method, PCR is conducted using a specific nucleic acid probe according to the present invention, and a change in fluorescence emission from the fluorescent dye after a reaction relative to fluorescence emission from the fluorescent dye before the reaction is determined in real time.

The term "PCR" as used herein means a variety of PCR methods. Examples can include RT-PCR, RNA-primed PCR, stretch PCR, reverse PCR, PCR making use of an Alu sequence, multiple PCR, PCR making use of a mixed primer, and PCR making use of PNA. Further, the term "quantitative" means, in addition to quantitation in general sense, quantitation of such an extent as detection as described above.

As described above, the term "target nucleic acid" as used herein means a nucleic acid the existing amount of which is intended to be determined, irrespective whether it is in a purified form or not and further irrespective of its concentration. Various other nucleic acids may also exist together with the target nucleic acid. For example, the target nucleic acid may be a specific nucleic acid in a co-cultivation

system microorganisms (a mixed system of RNAs or gene DNAs of plural microorganisms) or a symbiotic cultivation system of microorganisms (a mixed system of RNAs or gene DNAs of plural animals, plants and/or microorganisms), the amplification of which is intended. Purification of the target nucleic acid, if needed, can be conducted by a method known per se in the art. For example, purification can be effected using a purification kit or the like available on the market.

The conventionally-known quantitative PCR methods individually amplify, in the presence of Mg ions, a target nucleic acid by using dATP, dGTP, dCTP, dTTP or dUTP, a target nucleic acid (DNA or RNA), Taq polymerase, a primer, and a nucleic acid labeled with a fluorescent dye or an intercalator while repeatedly changing the temperature between low and high levels, and monitor increases in fluorescence emission from the fluorescent dye in real time in the course of the amplification [Jikken Igaku (Laboratory Medicine), 15(7), 46-51, Yodosha (1997)].

On the other hand, the quantitative PCR method according to the present invention is characterized in that the target nucleic acid is amplified by using the nucleic probe of the present invention and a change in fluorescence emission from the fluorescent dye, specifically, an increase in fluorescence emission in the case of the fluorescence emitting probe or a decrease in fluorescence emission in the case of the

fluorescence quenching probe is determined. The number of bases in a preferred probe of the present invention for use in the quantitative PCR according to the present invention may be from 5 to 50, preferably from 10 to 25, notably from 15 to 20.

5 No particular limitation is imposed on the probe insofar as it hybridizes to amplification products of the target nucleic acid in PCR cycles. The probe may be designed in either a forward type or a reverse type.

For example, the following designs can be mentioned when  
10 the nucleic acid probe is a fluorescence emitting probe. The above-described fluorescence emitting probes are all usable. Most suitable ones are those not labeled at the 3' ends for the reasons to be mentioned next. As the probe is used as a primer, the amount of the target nucleic acid labeled with the

15 fluorescent dye, namely, the quencher substance and fluorescent dye increases with the cycle of the reaction, so that the intensity of fluorescence in the reaction system at the time of the hybridization increases with the cycle of the reaction. Needless to say, those labeled at the 3' end can also be used  
20 sufficiently. In this case, they can be used as simple nucleic acid probes.

The followings can be mentioned as illustrative examples of the fluorescence quenching probe:

(1) A probe labeled, at an portion, preferably, an end  
25 thereof, with a fluorescent dye useful in the practice of the

present invention. The base sequence of the probe is designed such that, when hybridizes to a target nucleic acid, at least one G (guanine) base exists in the base sequence of the target nucleic acid at a position 1 to 3 bases apart from the end base of the target nucleic acid hybridized on the end portion or end  
5 of the probe where the probe is labeled with the fluorescent dye.

(2) A probe similar to the probe (1) except that the probe is labeled at the 3' end thereof with the fluorescent dye.

10 (3) A probe similar to the probe (1) except that the 5' end thereof with the fluorescent dye.

(4) A nucleic acid probe the base sequence of which is designed such that, when the probe hybridizes to a target nucleic acid, plural base pairs in a probe-nucleic acid hybrid complex form at least one G (guanine) and C (cytosine) pair at  
15 the end portion.

(5) A probe similar to the probe (4), wherein the probe has G or C as a 3' end base and is labeled at the 3' end thereof with a fluorescent dye.

20 (6) A probe similar to the probe (4), wherein the probe has G or C as a 5' end base and is labeled at the 5' end thereof with a fluorescent dye.

(7) A probe similar to any one of the probes (1) - (6) except that the OH group on the C atom at the 3'-position of ribose or deoxyribose at the 3' end or the OH group on the C atom at  
25

the 3'- or 2'-position of ribose at the 3' end has been phosphorylated.

(8) a nucleic acid probe labeled with the fluorescent dye at a modification portion other than the phosphate group on the 5' end or the OH group on the 3' end and having a base sequence designed such that, when the probe hybridizes to a target nucleic acid, plural base pairs in a probe-nucleic acid hybrid complex form at least one G (guanine) and C (cytosine) pair at the modification portion.

(9) a nucleic acid probe similar to any one of the probe(s) (1)-(6) except that the oligonucleotide of the probe has been chemically modified.

(10) A nucleic acid probe the base sequence of which is designed such that, when the probe hybridizes to a target nucleic acid, plural base pairs in a probe-nucleic acid hybrid complex form at least one G (guanine) and C (cytosine) pair and the G or C forming the base pair is modified by a fluorescent dye at a position other than the phosphate group on the 5' end or the OH group on the 3' end.

In the case of the probe (6), the 3' or 5' end may not be designed to G or C due to the base sequence of a target nucleic acid. If this should be the case, 5'-guanylic acid or guanosine or 5'-cytidylic acid or cytidine may be added to the 5' end of an oligonucleotide designed as a primer from the base sequence of the target nucleic acid. The probe so obtained can still

achieve the objects of the present invention adequately. The objects of the present invention can also be adequately achieved by adding 5'-guanylic acid or 5'-cytidylic acid to the 3' end. The expression "nucleic acid probe designed such that the 3' end or 5' end base thereof becomes G or C" as used herein is, therefore, defined to embrace not only probes designed based on the base sequence of the target nucleic acid but also probes added at the 5' end thereof with 5'-guanylic acid or 5'-cytidylic acid or guanosine or 5'-cytidylic acid or cytidine and probes added at the 5' end thereof with 5'-guanylic acid or 5'-cytidylic acid.

In particular, the above-described probe (7) of the present invention is designed such that it is not used as a primer. PCR is conducted by using a single probe of the present invention as opposed to two (fluorescent-dye-labeled) probes needed in a real-time quantitative PCR method making use of the FRET phenomenon. The probe is added to a PCR reaction system, and PCR is then conducted. During a nucleic acid extending reaction, the probe which has been in a form hybridized with the target nucleic acid or amplified target nucleic acid is degraded by polymerase and is dissociated off from the nucleic acid hybrid complex. The intensity of fluorescence of the reaction system at this time or the reaction system in which a nucleic acid denaturing reaction has completed is measured. Further, the intensity of fluorescence of the reaction system in which the target nucleic acid or amplified target nucleic acid has

hybridized with the probe (i.e., the reaction system at the time of an annealing reaction or at the time of the nucleic acid extending reaction until the probe is eliminated from the nucleic acid hybrid complex by polymerase). By calculating a decrease of the latter fluorescence intensity from the former fluorescence intensity, the concentration of the amplified nucleic acid is determined. The intensity of fluorescence is high when the probe has completely dissociated from the target nucleic acid or amplified target nucleic acid by the nucleic acid denaturing reaction or when the probe has been degraded out from the hybrid complex of the probe and the target nucleic acid or amplified nucleic acid at the time of extension of the nucleic acid. However, the intensity of fluorescence of the reaction system in which an annealing reaction has been completed and the probe has fully hybridized to the target nucleic acid or amplified target nucleic acid or of the reaction system until the probe is degraded out of the hybrid complex of the probe and the target nucleic acid or amplified target nucleic acid by polymerase at the time of a nucleic acid extending reaction is lower than the former. The decrease in the intensity of fluorescence is proportional to the concentration of the amplified nucleic acid.

In this case, the base sequence of the probe (7) may desirably be designed such that the  $T_m$  of a nucleic acid hybrid complex, which is available upon hybridization of the probe with

the target nucleic acid, falls within a range of the  $T_m$  value of the hybrid complex of the primer  $\pm 15^\circ\text{C}$ , preferably  $\pm 5^\circ\text{C}$ . If the  $T_m$  of the probe is lower than (the  $T_m$  value of the primer  $- 5^\circ\text{C}$ ), especially (the  $T_m$  value of the primer  $- 15^\circ\text{C}$ ), the probe does not hybridize so that no decrease takes place in the fluorescence emission from the fluorescent dye. If the  $T_m$  of the probe is higher than (the  $T_m$  value of the primer  $+ 5^\circ\text{C}$ ), especially (the  $T_m$  value of the primer  $+ 15^\circ\text{C}$ ), the probe also hybridizes to nucleic acid or acids other than the target nucleic acid so that the specificity of the probe is lost.

The probes other than the probe (7), especially the probe (6) is added as a primer to PCR reaction systems. Except for the PCR method according to the present invention, no PCR method is known to make use of a primer labeled further with a fluorescent dye. As the PCR reaction proceeds, the amplified nucleic acid is progressively labeled with the fluorescent dye useful in the practice of the present invention. Accordingly, the intensity of fluorescence of the reaction system in which the nucleic acid denaturing reaction has completed is high but, in the reaction system in which the annealing reaction has completed or the nucleic acid extending reaction is proceeding, the intensity of fluorescence of the reaction system is lower than the former intensity of fluorescence.

The PCR reaction can be conducted under similar conditions as in conventional PCR methods. It is, therefore,

possible to conduct amplification of a target nucleic acid in a reaction system the concentration of Mg ions in which is low (1 to 2 mM). Needless to say, the present invention can also be conducted even in a reaction system in which Mg ions are contained at such a high concentration (2 to 4 mM) as that employed in the conventionally-known quantitative PCR methods.

In the PCR method according to the present invention,  $T_m$  value can be determined by conducting the PCR of the present invention and then analyzing the melting curve of the nucleic acid with respect to the amplification products. This method is a novel analysis method of a melting curve of a nucleic acid. In this method, the nucleic acid probe employed as a nucleic acid probe or primer in the PCR method of the present invention can be used suitably.

In this case, designing of the base sequence of the probe according to the present invention into a sequence complementary with a region containing SNP (single nucleotide polymorphism) makes it possible to detect SNP from a difference, if any, in a dissociation curve of the nucleic acid from the probe of the present invention by analyzing the dissociation curve after completion of PCR. If a base sequence complementary with an SNP-containing sequence is used as a sequence for the probe of the present invention, a  $T_m$  value available from a dissociation curve between the sequence of the probe and the SNP-containing sequence becomes higher than a  $T_m$  value

available from a dissociation curve between the sequence of the probe and the SNP-free sequence.

③ Data analysis method

5 The present invention, in the third aspect thereof, relates to the method for analyzing data obtained by the above-described real-time quantitative PCR method.

10 A real-time quantitative PCR method is now practiced in real time by a system which is composed of a reactor for conducting PCR, an equipment for detecting fluorescence emission from a fluorescent dye, a user interface, namely, a computer-readable recording medium with various procedures of a data analysis method recorded as a program (also called "sequence detection software system"), and a computer for controlling them and analyzing data. Determination by the present invention is also conducted by such a system.

15 A description will first be made of an analyzer for real-time quantitative PCR. Any system can be used in the present invention insofar as it can monitor PCR in real time. Particularly suitable examples can include "ABI PRISM™ 7700 Sequence Detection System (SDS 7700)" (manufactured by 20 Perkin-Elmer Applied Biosystems, Inc., U.S.A.) and "LightCycler™ System" (manufactured by Roche Diagnostics, Mannheim, Germany).

25 The above-described reactor is an apparatus for repeatedly conducting a thermal denaturing reaction of a target

nucleic acid, an annealing reaction and an extending reaction of the nucleic acid (these reactions can be repeatedly conducted, for example, by successively changing the temperature to 95°C, 60°C and 72°C. The detection system comprises a fluorescence emitting argon laser, a spectrograph and a CCD camera. Further, the computer-readable recording medium with the various procedures of the data analysis method recorded as the program is used by installing it in the computer, and contains a program recorded therein for controlling the above-described system via the computer and also for processing and analyzing data outputted from the detection system.

The data analysis program recorded in the computer-readable recording medium comprises the following steps: measuring the intensity of fluorescence cycle by cycle, displaying each measured fluorescence intensity as a function of cycles, namely, as a PCR amplification plot on a display of the computer, calculating a threshold cycle number (Ct) at which the intensity of fluorescence is begun to be detected, forming a working line useful in determining from Ct values the number of copies of the nucleic acid in the sample, and printing data and plot values in the respective steps. When PCR is exponentially proceeding, a linear relationship is established between the logarithm of the number of copies of the target nucleic acid at the time of initiation of PCR and Ct. It is therefore possible to calculate the number of copies of the

target nucleic acid at the time of initiation of PCR by forming a working line based on known copy numbers of the target nucleic acid and detecting the Ct of a sample which contains the target nucleic acid the number of copies of which is unknown.

5           The PCR-related invention such as the above-described data analysis method is an invention for analyzing data obtained by such a real-time quantitative PCR method as described above. Its respective features will be described hereinafter. A first feature resides in a processing step for correcting a  
10           fluorescence intensity of a reaction system, which is measured when the nucleic acid amplified in each cycle is conjugated with the fluorescent dye or when the amplified nucleic acid hybridizes to a nucleic acid probe according to the present invention in the method for analyzing data obtained by the  
15           real-time quantitative PCR method, by a fluorescence intensity of the reaction system as obtained when the above-described conjugate of the fluorescent dye and the nucleic acid or the fluorescent dye-nucleic acid conjugate or the above-described hybrid complex of the nucleic acid probe of the present  
20           invention and the target nucleic acid or the nucleic acid hybrid complex has dissociated in each cycle, namely, the first feature resides in a correction-processing step.

          As a specific example of "the reaction system ..... when the amplified target nucleic acid is conjugated with the  
25           fluorescent dye or when the amplified target nucleic acid

hybridizes to a nucleic acid probe according to the present invention", a reaction system upon conducting a nucleic acid extending reaction or annealing at 40 to 85°C, preferably 50 to 80°C in each cycle of PCR can be mentioned. The actual temperature depends upon the length of the amplified nucleic acid.

Further, "the reaction system ..... when the above-described fluorescent dye-nucleic acid conjugate or the above-described nucleic acid hybrid complex has dissociated" can be a reaction system upon conducting thermal denaturation of the nucleic acid in each cycle of PCR, specifically at a reaction temperature of from 90 to 100°C, preferably 94 to 96°C. Illustrative is a system in which the reaction has been completed.

Any correction processing can be used as the correction processing in the correction processing step insofar as it conforms with the objects of the present invention. Specifically, correction processing including a processing step by the following formula (1) or formula (2) can be exemplified.

$$f_n = f_{\text{hyb},n} / f_{\text{den},n} \quad (1)$$

$$f_n = f_{\text{den},n} / f_{\text{hyb},n} \quad (2)$$

where

$f_n$ : correction-processed value in an  $n^{\text{th}}$  cycle as calculated in accordance with the formula (1) or formula (2),

$f_{\text{hyb},n}$ : intensity value of fluorescence of the reaction system available after the amplified nucleic acid has conjugated to the fluorescent dye or the amplified nucleic acid has hybridized to the nucleic acid probe labeled with the fluorescent dye in the  $n^{\text{th}}$  cycle, and

$f_{\text{den},n}$ : intensity value of fluorescence of the reaction system available after the fluorescent dye-nucleic acid conjugate or the nucleic acid hybrid complex has dissociated in the  $n^{\text{th}}$  cycle.

This step includes a sub-step in which correction-processed values obtained by the above-described processing are displayed on a computer display and/or the correction-processed values are likewise displayed and/or printed in the form of a graph as a function of cycles.

A second feature resides in a data analysis method, which comprises:

introducing correction-processed values, which have been calculated in accordance with the formula (1) or formula (2) in individual cycles, into the following formula (3) or formula (4) to calculate rates or percentages of changes in fluorescence between samples in the individual cycles:

$$F_n = f_n / f_a \quad (3)$$

$$F_n = f_o / f_n \quad (4)$$

where

$F_n$ : rate or percentage of a change in fluorescence in an  $n^{\text{th}}$

cycle as calculated in accordance with the formula (3)  
or formula (4),

$f_n$ : correction-processed value calculated in the  $n^{\text{th}}$  cycle  
as calculated in accordance with the formula (1) or  
formula (2), and

$f_a$ : correction-processed value calculated in a given cycle  
before a change in  $f_n$  is observed as calculated in  
accordance with the formula (1) or formula (2), and in  
general, a correction-processed value, for example, in  
one of  $10^{\text{th}}$  to  $40^{\text{th}}$  cycles, preferably one of  $15^{\text{th}}$  to  $30^{\text{th}}$   
cycles, more preferably one of  $20^{\text{th}}$  to  $30^{\text{th}}$  cycles is  
adopted; and

comparing the rates or percentages of changes in  
fluorescence.

This step includes a sub-step in which calculated values  
obtained by the above-described processing are displayed on a  
computer display and/or are printed or the calculated values  
are likewise displayed and/or printed in the form of a graph  
as a function of cycles. This sub-step may be applied or may  
not be applied to the correction-processed values obtained by  
the formula (1) or formula (2).

A third feature resides in a data analysis method, which  
comprises the following processing steps:

1) performing processing in accordance with the following  
formula (5), (6) or (7) by using data of rates or percentages

of changes in fluorescence as calculated in accordance with said formula (3) or (4):

$$\log_b(F_n), \ln(F_n) \quad (5)$$

$$\log_b\{(1-F_n) \times A\}, \ln\{(1-F_n) \times b\} \quad (6)$$

$$5 \quad \log_b\{(F_n-1) \times A\}, \ln\{(F_n-1) \times A\} \quad (7)$$

where

A,b: desired numerical values, preferably integers, more preferably natural numbers and, when A=100, b=10,  $\{(F_n-1) \times A\}$  is expressed in terms of percentage (%), and

10  $F_n$ : rate or percentage of a change in fluorescence in an  $n^{\text{th}}$  cycle as calculated in accordance with the formula (3) or formula (4),

2) determining a cycle in which said processed value of said processing step 1) has reached a constant value,

15 3) calculating a relational expression between cycle of a nucleic acid sample of a known concentration and the number of copies of said target nucleic acid at the time of initiation of a reaction, and

20 4) determining the number of copies of said target nucleic acid in an unknown sample upon initiation of PCR.

Preferably, these steps are performed in the order of 1) → 2) → 3) → 4).

Each of these steps 1) to 3) may include a sub-step in which processed values obtained by the corresponding processing are displayed on a computer display and/or the processed values

25

are likewise displayed and/or printed in the form of a graph as a function of cycles. The step 4) should include at least a printing sub-step as the processed values obtained in the step 4) have to be printed, although the processed values obtained in the step 4) may also displayed on a computer display.

Incidentally, the correction-processed values obtained by the formula (1) or (2) and the calculated values obtained by the formula (3) or (4) may be or may not be displayed on a computer display and/or printed in the form of graphs as a function of cycles, respectively. These displaying and/or printing sub-steps may, therefore, be added as needed.

The above-described data analysis method is particularly effective when decreases in fluorescence emission from the fluorescent dye are measured in the real-time quantitative PCR method, that is, when fluorescence quenching probes are used. As a specific example, the real-time quantitative PCR method according to the present invention, which makes use of a fluorescence quenching probe, can be mentioned.

A fourth feature resides in an analysis system for real-time quantitative PCR, which comprises processing and storing means for performing a data analysis method for the above-described real-time quantitative PCR method of the present invention.

A fifth feature resides in a computer-readable recording medium with individual procedures of a data analysis method,

which is adapted to analyze PCR by using the analysis system for the real-time quantitative PCR, stored as a program therein, wherein the program is designed to make a computer perform the individual procedures of the data analysis method of the present invention.

A sixth feature resides in a novel method for determining a nucleic acid, which comprises using the data analysis method, determination and/or analysis system and/or recording medium of the present invention in the nucleic acid determination method.

A seventh feature resides in a method for analyzing data obtained by the above-described method of the present invention for the analysis of a melting curve of a nucleic acid, namely, data obtained by the method of the present invention in which the  $T_m$  value of the nucleic acid is determined by conducting PCR.

Specifically, the seventh feature resides in an analysis method, which comprises the following steps: gradually heating a nucleic acid, which has been amplified by the PCR method of the present invention, from a low temperature until complete denaturation of the nucleic acid (for example, from 50°C to 95°C; measuring an intensity of fluorescence at short time intervals (for example, at intervals equivalent to a temperature rise of from 0.2°C to 0.5°C) during the heating step; displaying results of the measurement as a function of time on a display, namely,

a melting curve of the nucleic acid; differentiating the melting curve to obtain differentiated values ( $-dF/dT$ , F: intensity of fluorescence, T: time); displaying the differentiated values as derivatives on the display; and determining a point of inflection from the derivatives. In the present invention, the intensity of fluorescence increases as the temperature rises. Preferable results can be obtained in the present invention by adding to the above-described step a further processing step in which in each cycle, the intensity of fluorescence at the time of the nucleic acid extending reaction, preferably at the time of completion of the PCR reaction is divided by the value of fluorescence intensity at the time of the thermal denaturing reaction.

A measurement and/or analysis system for the real-time quantitative PCR of the present invention, said real-time quantitative PCR including the method of the present invention for the analysis of the melting curve of a nucleic acid added to the above-described novel method of the present invention for the analysis of data obtained by a PCR method, also falls within the technical scope of the present invention.

A still further feature of the present invention resides in a computer-readable recording medium with the individual procedures of the method of the present invention for the analysis of the melting curve of a nucleic acid recorded therein as a program such that the procedures can be performed by a

computer or a computer-readable recording medium with the individual procedures of the method of the present invention for the analysis of data obtained by a PCR method recorded therein as a program such that the procedures can be performed  
5 by a computer, wherein a program designed to make the computer perform the individual procedures of the method of the present invention for the analysis of the melting curve of the nucleic acid is additionally recorded.

The above-described data analysis methods, systems and recording media of the present invention can be used in a variety  
10 of fields such as medicine, forensic medicine, anthropology, paleontology, biology, genetic engineering, molecular biology, agricultural science and phytobreeding. They can be suitably applied to microorganism systems called "co-cultivation  
15 systems of microorganisms" or "symbiotic cultivation systems of microorganisms", in each of which various kinds of microorganisms are contained together or a microorganism and other animal- or plant-derived cells are contained together and cannot be isolated from each other. The term "microorganisms"  
20 as used herein means microorganisms in general sense, and no particular limitation shall be imposed thereon. Illustrative are eukaryotic microorganisms, prokaryotic microorganisms, mycoplasmas, virus and rickettsias.

The vial count of a particular cell strain in a co-  
25 cultivation system of microorganisms or a symbiotic cultivation

systems of microorganisms can be determined by determining the number of copies of the 5S rRNA, 16S rRNA or 23S rRNA of the particular cell strain or its gene DNA in the system by using one or more of the above-described data analysis methods, systems and recording media of the present invention, because the number of copies of the gene DNA of 5S rRNA, 16S rRNA or 23S rRNA is specific to each cell strain. In the present invention, the vial count of a particular cell strain can also be determined by applying the real-time quantitative PCR of the present invention to a homogenate of a co-cultivation system of microorganisms or a symbiotic cultivation systems of microorganisms. It shall also be noted that this method also falls with the technical scope of the present invention.

#### ④ Polymorphous analysis method

The feature of the polymorphous analysis method according to the present invention resides in the use of the nucleic acid probe of this invention in a conventional polymorphous analysis method to determination a nucleic acid. The term "polymorphous" or "polymorphism" as used herein means biological polymorphous or polymorphism. In the present invention, it means especially the polymorphism of a gene (RNA, DNA, gene) on which the polymorphism is brought about. It has the same meaning as commonly employed these days in molecular biology.

The term "polymorphous analysis" means to analyze and/or

determine what polymorphism a gene has.

Currently-available examples of the conventional polymorphous method include SSOP (sequence specific oligonucleotide probe) method, RELP (restriction fragment length polymorphism) method, T-RELP (terminal restriction fragment length polymorphism) method, SSCP (single strand conformation) method, MPH method, CFLP (cleavage fragment length polymorphism) method, SSP (sequence specific primer) method, PHFA (preferential homoduplex formation assay) method, SBT (sequence base typing) method [PCT Ho, Riyo no Tebiki (PCR Methods, Manual for Their Use), Chugai Medical Publishing Co., Ltd. (1998); Tanpakushitsu, Kakusan, Koso (Proteins, Nucleic Acids, Enzymes), 35(17), KYORITSU SHUPPAN CO., LTD. (1990); Jikken Igaku (Laboratory Medicine), 15(7) (special number), Yodosha (1997)]. T-RELP method or CFLP method can be especially suitably applied, although the methods currently used in polymorphous analyses are all usable in the present invention

Features of the polymorphous analysis method will hereinafter be described specifically in order.

The first feature resides in a quantitative gene amplification method making use of the nucleic acid probe of this invention. Any quantitative gene amplification method can be adopted insofar as it has quantitateness. For example, PCR methods can be adopted suitably. Among these, quantitative PCR methods and real-time monitoring, quantitative PCR

methods are more preferred.

Examples of conventionally-known, quantitative PCR methods can include RT-PCR, RNA-primed PCR, Stretch PCR, reversed PCR, PCT making use of an Alu sequence, multiple PCR, PCR making use of a mixed primer, and PCR making use of PNA.

According to these conventionally-known, quantitative PCR methods, a target gene is amplified by cycling the temperature between a low temperature and a high temperature in the presence of Mg ions while using dATP, dGTP, dCTP and dTTP or dUTP, a target gene (DNA or RNA), Taq polymerase, a primer and a nucleic acid probe labeled with a fluorescent dye or an intercalator, and an increase in the emission of fluorescence from the fluorescent dye in the course of the amplification is monitored in a real-time manner [Jikken Igaku (Laboratory Medicine), 15(7), 46-51, Yodosha (1997)].

The quantitative PCR method according to the present invention, which makes use of the invention nucleic acid probe, is a method in which the probe labeled with the fluorescent dye is used. It is a quantitative PCR method that makes use of a probe designed such that the intensity of fluorescence from the fluorescent dye changes (specifically, increases in the case of a fluorescence emitting probe or decreases in the case of a fluorescence quenching probe) when the probe hybridizes to a target nucleic acid.

For example, as has been described in detail in connection

with the second aspect of the invention, a fluorescence quenching probe is labeled at an end thereof with a fluorescent dye, and its base sequence is designed such that, when the probe hybridizes at the end portion thereof to a target gene, at least one G (guanine) base exists in a base sequence of the target gene at a position 1 to 3 bases apart from the portion of an end base pair of the target gene hybridized with the probe, whereby the fluorescent dye is reduced in fluorescence emission when the probe hybridizes to the target gene.

Preferably, the fluorescence quenching probe is labeled at the end thereof with the fluorescent dye, and its base sequence is designed such that, when the probe hybridizes to the target gene, base pairs of the hybrid complex of the probe and the target gene forms at least one G (guanine) and C (cytosine) pair (GC base pair) at the end thereof, whereby the fluorescent dye is reduced in the intensity of fluorescence when the probe hybridizes to the target gene.

If the 5' or 3' end cannot be designed to G or C due to the base sequence of a target gene, the objects of the present invention can also be adequately achieved by adding 5'-guanylic acid or 5'-cytidylic acid to the 5' end of an oligonucleotide designed as a primer from the base sequence of the target nucleic acid. The expression "fluorescence quenching probe" is, therefore, defined to embrace not only probes designed based on the base sequence of the target nucleic acid but also probes

added at the 3' or 5' ends thereof, preferably the 5' ends thereof with 5'-guanylic acid or 5'-cytidylic acid.

If it is inconvenient to form the end into C or G, similar fluorescence quenching effect can also be obtained by  
5 fluorescence labeling C or G in the chain of a probe or primer.

The nucleic acid probe of this invention to be used contains 5 to 50 bases, preferably 10 to 25 bases, especially preferably 15 to 20 bases. No particular limitation is imposed on its base sequence insofar as the probe hybridizes  
10 specifically to the target gene.

According to the quantitative PCR method making use of the fluorescence quenching probe, the target gene can be easily and specifically amplified in short time. When a fluorescence quenching probe labeled at the 5' end thereof with a fluorescent dye is used, a target gene labeled at the 5' end thereof with the fluorescent dye is amplified [Jikken Igaku (Laboratory  
15 Medicine), 15(7), Yodosha (1997)].

As a thermal cycler for use in the quantitative PCR method, any one of various equipment currently available on the market  
20 can be conveniently used no matter whether or not it permits real-time monitoring. Particularly preferred examples of equipment, which permit real-time monitoring, can include "ABI PRISM™ 7700 Sequence Detection System" (SDS 7700) (trade name; manufactured by Perkin-Elmer Applied Biosystem, Inc., CA,  
25 U.S.A.) and "LightCycler™ System" (trade name; manufactured

by Roche Diagnostic GmbH, Mannheim, Germany).

Amplification of a gene can be attained under amplifying reaction conditions known to date. It is generally desired to proceed with amplification to an amplification degree which is commonly used. In the course of the amplification of the target gene, the intensity of fluorescence is measured by a fluorimeter. Changes in the intensity of fluorescence are proportional with amplified amounts of the gene. Plotting of the changes in the intensity of fluorescence as a function of time (cycles in the case of PCR) on an ordinary graph paper gives an S-shaped (sigmoid) curve, whereas their plotting on a semilog graph paper gives a line, which linearly increases in the beginning like an exponential function but then forms a curve which reaches a gentle plateau.

As the degree of amplification of the target gene, in other words, the time to stop the amplifying reaction of the gene to improve the quantitateness of the initial amount of the gene before starting PCR depends upon the purpose of the polymorphous analysis, no particular limitation is imposed thereon. Described specifically, when a polymorphous system is analyzed for only priority polymorphism, it is suited to amplify the target gene for a desired time from the initial observation of a change in the intensity of fluorescence until before the above-described plateau is reached. It is most preferable to stop the reaction in an exponential growth phase

[i.e., before reaching a midpoint of the sigmoid curve (a point where a derivative of the curve becomes 0)]. When it is desired to analyze all polymorphous species contained in the polymorphous system, it is desired to conduct several experiments in a trial and error manner to determine a degree of amplification considered to be the best and then to amplify the gene to such extent that genes, which show polymorphism in the reaction system, can all be observed. A method - in which amplification is conducted by dividing it in plural stages, in other words, an experiment is conducted at plural degrees of amplification and the results are analyzed as a whole - can also be adopted appropriately, because minor polymorphous species tend to draw a sigmoid curve having large time lags.

When the quantitative PCR method, especially the real-time monitoring quantitative PCR method is performed using the fluorescence quenching probe of this invention as a primer, the fluorescence quenching probe as the primer is used repeatedly for the amplification of the target gene so that the target gene labeled at the 5' end thereof with the fluorescent dye is amplified. The amplified target gene then hybridizes to the corresponding target gene. When this hybridization takes place, the intensity of fluorescence decreases. It is therefore only required to conduct the amplifying reaction to the best degree of amplification in a similar manner as described above while tracing decreases in the intensity of

fluorescence. This quantitative PCR method can also be conducted under similar reaction conditions as the conventional PCR methods. Accordingly, amplification of a target gene can be conducted in a reaction system the Mg ion concentration of which is low (1 to 2 mM) or, as was known conventionally, is high (2 to 4 mM).

It is preferred to prepare a working line for the target gene by using a target gene before the amplifying reaction of the target gene. A description will now be made about an illustrative case in which the above-described fluorescence quenching probe was used as a primer and the real-time monitoring quantitative PCR method was conducted.

Plotting of decreases in the intensity of fluorescence as a function of cycles on an ordinary graph paper gives an S-shaped (sigmoid) curve. An exponential relation exists between the number of cycles at a time point where the rate of decrease was the greatest and the initial number of copies of the target gene (the number of copies before the initiation of PCR), that is, the target gene in the initial stage. Advanced preparation of a target straight line, which represents the correlation between the number of cycles and the number of copies at that time point, makes it possible to determine the initial number of copies of the target gene in an unknown sample, namely, the initial amount of the target gene.

Incidentally, the above-described quantitative PCR

method making use of the fluorescence quenching probe is a novel method developed by the present inventors.

As the second feature of the quantitative polymorphous analysis method, it is an analysis method for analyzing data obtained by the quantitative PCR method.

As a matter of fact, it is nothing but a method for analyzing data obtained by the above-described quantitative PCR process. This analysis method is currently most suited for determining the initial amount of the target gene as accurately as possible.

This invention also relates to a reagent kit for use in the above-described quantitative gene amplification process and also to a computer-readable recording medium characterized in that a program for making a computer perform the above-described data analysis method is recorded.

Moreover, the present invention also relates to a data analysis system characterized in that the system is provided with means for conducting the above-described data analysis method.

The third feature of the present invention relates to a method for analyzing polymorphism with respect to genes amplified by the quantitative PCR method according to the present invention.

Now, this polymorphous analysis method will be described specifically. Among various polymorphous analysis methods,

T-RFLP can be suitably used in the present invention. As an example of the present invention, a gene is amplified by a quantitative PCR method making use of a fluorescence quenching probe as a primer, especially by a real-time monitoring quantitative PCR method, and the initial amount of the gene before PCR is determined. Further, a detailed description will be made about a method for analyzing polymorphism of the amplification products by T-RFLP. Incidentally, the gene amplified by using the fluorescence quenching probe as a primer is labeled at the 5' end thereof with the fluorescent dye useful in the practice of the present invention.

(1) Firstly, the amplification products are digested by a restriction endonuclease. As this restriction endonuclease, the currently known restriction endonucleases are all usable. Illustrative are Bco FI, Hha I, Hph I, Mnl I, Rca I, Alu I and Msp I. Among these, preferred are Rca I, Alu I and Msp I, with Hha I being most preferred. As digesting reaction conditions, conditions generally employed for the currently known genes can be used. If Hha I is chosen as a restriction endonuclease, for example, it is reacted at 37°C for 6 hours at a restriction endonuclease concentration of 10 units.

(2) Gene fragments digested as described above can preferably be thermally modified into single-stranded forms. This modification treatment can be conducted under usual conditions known to the public. For example, they are treated

at 97°C for 5 minutes and then chilled in ice.

(3) Analysis and determination of gene fragments

In the polymorphous analysis method of the present invention, only the gene fragments labeled with the fluorescent dye are analyzed and determined by electrophoresis, HPLC, sequencer or the like.

Described specifically, individual bands and band peaks are detected based on fluorescence intensities. This detection can be conducted using an ordinary analyzer currently available on the market. Examples of the analyzer can include "ABI 373A" (trade name, a sequencer manufactured by Applied Biosystems Group, CT, U.S.A.), "ABI 377" (trade name, a sequencer manufactured by Applied Biosystems Group, CT, U.S.A.), and "Biofocus 3000" (trade name; manufactured by Bio-Rad Laboratories, Inc. CA, U.S.A.).

In the present invention, appearance of plural bands or plural peaks in the above-described analysis means existence of polymorphism. A single band or a single peak means non-existence of polymorphism. A fluorescence intensity ratio of individual bands or peaks obviously means a polymorphous ratio. As the amount of a target gene before PCR is determined in the quantitative PCR method of the present invention, multiplication of the determined value by the above-described polymorphous ratio makes it possible to determine the initial amounts of the individual species of the polymorphous gene.

A method for obtaining data with respect to polymorphism as described above has been successfully provided for the first time owing to the use of the quantitative PCR method making use of the fluorescence quenching probe of the present invention.

5 Further, a convenient reagent kit for quantitative polymorphous analysis can also be provided by either including or attaching a reagent kit for the quantitative PCR method.

10 In addition, additional recording of a program, which is adapted to make a computer perform an analysis of data of the above-described real-time monitoring quantitative PCR, in a computer-readable recording medium - in which a program for making the computer perform the analysis method of data obtained by the above-described polymorphous analysis method has already been recorded - can provide a more convenient, computer-  
15 readable recording medium for the analysis of data obtained by the quantitative polymorphous analysis method.

Moreover, combined arrangement of a data analyzer for PCR with a polymorphous analyzer equipped with means for performing the quantitative polymorphous analysis method can provide a  
20 more convenient polymorphous analyzer.

The present invention will next be described more specifically based on the following Examples and Comparative Examples. Examples 1-7 relate to fluorescence emitting probes according to the present invention.

25 Example 1

Synthesis of nucleic acid probe

Assuming that the base sequence of a target nucleic acid was (5')GGGGGGAAAAAAA(3') formed of an oligodeoxy-ribonucleotide, synthesis of a nucleic acid probe according to the present invention was conducted in the following order.

Designing of nucleic acid probe

As the base sequence of the target nucleic acid was (5')GGGGGGAAAAAAA(3'), it was possible to readily design the base sequence of the nucleic acid probe as (5')TTTTTTTTTCCCCC(3') formed of an oligodeoxyribonucleotide. The nucleic acid probe according to the present invention was designed further as will be described hereinafter. It was decided to label a fluorescent dye, Texas Red, to a phosphate group on the 5'end and a quencher substance, Dabcyl, to an OH group on the 6-C of a base ring of the 6<sup>th</sup> thymine from the 5'end (Design of Texas Red-(5')TTTTTT(Dabcyl-)TTTCCCCC(3')).

Using "5'Amino-Modifier C6 Kit" (trade name, product of Glen Research Corporation, VA, U.S.A.), the phosphate group of thymidylic acid was modified with an amino linker (protecting group: MMT). Using "Amino-Modifier C2dT Kit" (trade name, product of Glen Research Corporation, VA, U.S.A.), the OH group on the 6-C of the base ring of thymidine was modified with an amino linker (protecting group: TFA). Using those modified thymidylic acid and thymidine, an oligonucleotide having the following base sequence was synthesized by a DNA synthesizer

("ABI 394") (trade name, manufactured by PerkinElmer Japan Co., Ltd., Japan). Specifically, it was a deoxyribooligonucleotide having the base sequence of (5')TTTTTTTTTCCCCC(3'), the phosphate group on the 5' end was modified with the amino linker (protecting group MMT), and the OH group on the 6-C of the base ring of the 6<sup>th</sup> thymine from the 5' end was modified with the amino linker (protecting group: TFA). Incidentally, the synthesis of DNA was conducted by the  $\beta$ -cyanoethylphosphoramidate method. After the synthesis, elimination of the protecting groups was conducted with 28% aqueous ammonia at 55°C for 5 hours.

#### Purification of synthesized product

The synthetic oligonucleotide obtained as described above was dried into a dry product. The dry product was dissolved in 0.5 M  $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$  buffer (pH 9.0). The solution was subjected to gel filtration through "NAP-25 Column" (trade name, product of Pharmacia AB, Uppsala, Sweden), whereby unreacted substances were removed.

#### Labeling with quencher substance

The filtrate was dried into solid, and dissolved in sterilized water (150  $\mu\text{L}$ ) (oligonucleotide A solution). "Dabcyl-NHS" (trade name, product of Molecular Probes, Inc., OR, U.S.A.) (1 mg) was dissolved in DMF (dimethylformamide) (150  $\mu\text{L}$ ), and the oligonucleotide A solution and 1 M  $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$  buffer (150  $\mu\text{L}$ ) were added. The resulting mixture was stirred,

followed by a reaction overnight at room temperature.

Purification of synthesized product

The reaction product was subjected to gel filtration through "NAP-25" (trade name, product of Pharmacia AB, Uppsala, Sweden) to remove unreacted substances. Then, the protecting group (MMT) on the 5' end was eliminated with 2% TFA. Reversed phase HPLC was conducting using "SEP-PAC C<sub>18</sub> column" to fractionate the target product in which the quencher substance, Dabcyl" was bound to the linker  $-(CH_2)_6-NH_2$  of the oligonucleotide. The fractionated product was subjected to gel filtration through "NAP-10" (trade name, product of Pharmacia AB, Uppsala, Sweden).

Labeling with fluorescent dye

The gel filtrate was dried into solid, and dissolved in sterilized water (150  $\mu$ L) (oligonucleotide B solution). "Sulforhodamine 101 Acid Chloride" (trade name, product of Dojindo Laboratories, Kumamoto, Japan) (1 mg) was dissolved in DMF (100  $\mu$ L), and the oligonucleotide B solution and 1 M NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub> buffer (150  $\mu$ L) were added. The resulting mixture was stirred, followed by a reaction overnight at room temperature to have the fluorescent dye, Texas Red, bound to the amino linker on the 5' end.

Purification of synthesized product

The reaction product was subjected to gel filtration through "NAP-25" (trade name, product of Pharmacia AB, Uppsala,

Sweden) to remove unreacted substances. Reversed phase HPLC was conducted in a similar manner as described above, and a nucleic acid probe according to the present invention, which was an oligonucleotide with the quencher substance bound to the 7<sup>th</sup> thymine base from the 5' end and also with the fluorescent dye, Texas Red, added to the 5' end, namely, a nucleic acid probe labeled with the fluorescent dye and the quencher substance was obtained. Incidentally, the invention nucleic acid probe was eluted with a lag from the oligonucleotide with the quencher substance bound thereon.

Quantitation of the invention nucleic acid probe conducted by measuring a value at 260 nM with a spectrophotometer. With respect to the probe, scanning of an absorbance over 650 nm to 220 nm was also conducted using the spectrophotometer. As a result, absorptions ascribed to Dabcyl, Texas Red and DNA, respectively, were confirmed. Further, the purity of the purified product was tested by similar reversed phase HPLC as in the above. As a result, it was confirmed that the purified product gave a single peak.

The invention nuclear acid probe synthesized as described above is free of any base sequence having complementation at at least two positions between the base chains at positions where the probe was labeled with Texas Red as a fluorescent dye and Dabcyl as a quencher substance, respectively. The invention nuclear acid probe, therefore, does not form any

double-stranded chain in its own chain. In other words, the invention nuclear acid probe does not form any stem-loop structure.

The above-described reversed phase chromatography was conducted under the following conditions:

Eluting solvent A: 0.05 N TEAA 5% CH<sub>3</sub>CN

Eluting solvent B (for gradient elution): 0.05 N TEAA  
40% CH<sub>3</sub>CN

Column: "SEP-PAK C18" (trade name), 6 x 250 mm

Elution rate: 1.0 mL/min

Temperature: 40°C

Detection: 254 nm

## 実施例 2

### 標的核酸の合成：

前記のオリゴヌクレオチドの合成と同様にして、(5')GGGGGAAAAAAAAA(3')なる塩基配列のオリゴヌクレオチドを合成して、本発明の標的核酸とした。

【0159】

## 実施例 3

標的核酸に本発明のプロープをハイブリダイズさせた反応系の蛍光強度測定：

石英セル（10 mm×10 mm）（容量4 mL）に500  $\mu$ Lの緩衝液（2M NaCl、200 mM Tris-HCl：pH=7.2）を添加し、次に1460  $\mu$ Lの滅菌蒸留水を添加し攪拌した。そこに、8.0  $\mu$ Lの本発明の核酸プローブ（10  $\mu$ M）溶液を添加して攪拌した。35℃に保温して蛍光強度を時間を追って測定した（励起波長：581 nm（8 nm幅））、測定蛍光波長：603 nm（8 nm幅）。ついで、160 nM濃度の標的核酸溶液32.0  $\mu$ Lを添加し、攪拌した。そして時間を追って蛍光強度を前記と同じ条件で測定した。その結果を図1に示した。図から、標的核酸を添加したことにより、蛍光強度が増加し、増加度が極めて短時間すなわち100秒（1分40秒）以内に一定になることが分かる（なお、分子ビーコンの場合、約15分を必要とする：Nature Biotechnology、14巻、303～308ページ、1998年）。このことは本発明の核酸測定方法が短時間で実施できることを示している。

【0160】

実施例4

標的核酸の測定：

標的核酸の濃度を種々替える以外は前記と同じ条件で、各濃度について蛍光強度を測定した。その結果を図2に示した。図から、標的核酸の濃度に応じて蛍光強度も増加し、この関係が比例関係にあることが判明した。

以上の結果から、本発明の核酸プローブを用いれば、精度のよい核酸測定ができることが認識される。

【0161】

実施例5 蛍光色素とクエンチャー物質間の距離の関係

図3に示す塩基配列のデオキシリボオリゴヌクレオチドを実施例1と同様に合成した。そして実施例1と同様にプローブとしては、デオキシリボオリゴヌクレオチドの5'末端のリン酸基に蛍光色素Texas Redを標識し、チミン塩基の6位CのOH基にクエンチャー物質Dabcylを標識した。そして、標識チミン塩基を3'末端側に1塩基ずつ移動させた。このような本発明のプローブを20種合成した。各プローブを相補する標的デオキシリボオリゴヌクレオチ

ドにハイブリダイズさせて、ハイブリダイゼーション前後の蛍光強度の変化量を測定した。

#### 【0162】

石英セル（実施例3と同じ）に500 $\mu$ lのトリス緩衝液（2M NaCl、200mM Tris-HCl、pH7.2）を添加した。次に1460 $\mu$ lの滅菌蒸留水を添加して攪拌した。そこに、10 $\mu$ Mの本発明のプロープ溶液を8.0 $\mu$ l添加し、攪拌した（プロープの終濃度：40nM）。35℃に保温して蛍光測定を行った（励起：581nm、蛍光：603nm、スリット幅：8nm（両者とも））。ついで、10 $\mu$ Mの標的デオキシリボオリゴヌクレオチド溶液を32.0 $\mu$ lを添加し、攪拌した（標的デオキシリボオリゴヌクレオチドの終濃度：160nM）。そして、前記と同条件で蛍光測定を時間を追って行った。

#### 【0163】

図6にその結果を示す。この図からも明らかなように、DabcylとTexas Redで2重修飾した本発明の蛍光発光プロープは、ほとんどのプロープにおいて、標的デオキシリボオリゴヌクレオチドとハイブリダイズすると、ハイブリダイゼーション前の蛍光発色量に比較して、<sup>3/</sup>蛍光発色の増加が観察された。また、最大の蛍光発色はTexas Red標識リン酸基を有する塩基からDabcyl標識塩基の塩基間の距離（Texas Redで標識された塩基の塩基番号を0塩基とした場合）が6塩基のときにみられた。このときの蛍光発色量は約11倍であった。また、塩基間距離が16塩基のときにも大きな蛍光発色量が観察され、このときの蛍光発色量も6塩基同様に約11倍であった。DNAのラセンは、10塩基で1回転することから、5'末端塩基から見た6番目、16番目の塩基はほぼラセンの裏側になる。よって、6番目、16番目の塩基をクエンチャー物質でラベルすることで、一本鎖の時には、DabcylとTexas Red間の電子移動による蛍光消光が起っていたが、ハイブリダイゼーションにより物理的にDabcylとTexas Redが引き離された結果、電子移動による蛍光消光が解除され、Texas Redが蛍光発色したと考えられた。

#### 【0164】

### 実施例 6 蛍光色素と蛍光発色量の関係

本発明の蛍光発光プローブにおける蛍光色素の種類について検討した。実験は実施例 5 と同様に行った。ただし、蛍光色素と Dabcyl の塩基間距離を 6 塩基とした。また、蛍光測定のスリット幅を励起、測定とも 5 nm とした。

その結果を表 1 に示した。

クエンチャーである Dabcyl の吸収は、400–500 nm にあるが、蛍光発色量が多いプローブは、Dabcyl の吸収を大きく外れ、550 nm より長波長側に蛍光発色を出すプローブが多い。この現象は、550 nm より長波長側に蛍光を出す蛍光色素の場合、DABCYL の蛍光消光機構は主に、FRET ではなく光励起電子移動によるものと考えられる。プローブの立体構造の変化により、物理的に Dabcyl と蛍光色素が引き離されるため、光励起電子移動による蛍光消光現象が解除される。FITC など Dabcyl の吸収に近い波長の蛍光を出す蛍光色素の場合、立体構造の変化により物理的に、Dabcyl と蛍光色素が引き離され、光励起電子移動による蛍光消光現象が解除されても、FRET による蛍光消光が大部分を占めるため、FRET の蛍光消光からの蛍光発色あまり起らないと考えられた。したがって、本発明の蛍光発光プローブで用いる蛍光色素は以下の 3 点の条件をみたす色素が望ましい。すなわち、(1)

光励起電子移動による蛍光消光現象が Dabcyl の間で発生する蛍光色素である。(2) Dabcyl の吸収から大きく外れた波長の蛍光を発する蛍光色素である。(3) ハイブリ前の蛍光強度を押さえるため(光励起電子移動による蛍光消光現象がより起こりやすいように)、Dabcyl との相互作用の強い蛍光色素である。

【0165】

表 1. 各種蛍光色素と蛍光発色量との関係

蛍光色素	励起波長	蛍光波長	標的核酸添加前蛍光強度 (A)	標的核酸添加後蛍光強度 (B)	A/B
FITC	480	510	11.5	18.5	1.6
TET	500	530	36.8	48.0	1.3
HEX	520	540	3.9	7.4	1.9
Cy3	540	560	2.0	7.4	3.7
Bodipy 581/591	555	582	1.6	6.4	4.0
Alexa531	500	539	7.3	26.0	3.6
6-ROX	560	590	4.8	18.9	3.9
Alexa594	575	603	2.2	15.22	6.9
Bodipy TR	585	615	2.4	9.6	4.0
Texas red	585	603	2.1	21	10

## 【0166】

## 実施例7 蛍光色素、クエンチャーで鎖中塩基を修飾したプローブ

図5に示したような、蛍光色素とクエンチャーで鎖中の塩基を修飾したプローブと標的デオキシリボオリゴヌクレオチドを、以下の点を除いて実施例1と同様な方法で合成した。(1) 5'Amino-Modifier C6キット(Glen Research社製、米国)を用いる方法の替わりに、Amino-Modifier C6 dT(Glen Research社製、米国)を用いる方法にて、Texas Redをプローブに修飾した。(2) 5'Amino-Modifier C6キット(Glen Research社製、米国)を介してプローブを修飾する方法の替わり

に、Dabcyl dT(Glen Research社製、米国)を用いて、直接塩基鎖中に導入した。

(3) よって、Dabcyl修飾工程とその後の精製工程を省いた。

そしてそれが実際使用できるのかどうかを、実施例5と同様にして検討した。また、クエンチャー(Dabcyl)と蛍光色素(Texas Red)標識塩基間の距離の影響をも検討した。図8にその結果を示す。この結果からも明らかなように、蛍光色素、クエンチャーで鎖中の塩基を修飾したプローブでも、実際使用できることが判明した。また、最大の蛍光発色は、5'末端のリン酸基にTexas Redを修飾したものと同様に、Texas RedとDABCYLとの塩基間の距離が6塩基、16塩基のときにみられた。このときの蛍光発色量はハイブリダイゼーション前に比較して約10倍であった。

#### 【0167】

実施例8から31及び比較例1は本発明の蛍光消光プローブに関するものである。

#### 実施例8

大腸菌 (*Escherichia coli*) の16SrRNAの核酸塩基配列にハイブリダイズする、即ち、(3')CCGCTCACGC ATC(5')の塩基配列を有する核酸プローブの調製を以下の通りに行った。

#### 【0168】

核酸プローブの調製：(3')CCGCTCACGC ATC(5')の塩基配列をもつオリゴデオキシリボヌクレオチドの3'末端のデオキシリボースの3'位炭素のOH基に、 $-(CH_2)_7-NH_2$ を結合したものを、メドランド・サーティファイド・レージント・カンパニー社 (Midland Certified Reagent Company、米国) から購入した。更に、モレキュラープローブ (Molecular Probes) 社からフロオ・リポーターキット (FluoReporter Kit) F-6082 (ボデビーFLのプロピオン酸サクシニミジルエステル (BODIPY FL propionic acid succinimidyl ester) の他に、当該化合物をオリゴヌクレオチドのアミン誘導体に結合する試薬を含有するキット) を購入した。当該キットを前記購入のオリゴヌクレオチドに作用させて、本実施例で使用するボデビーFLで標識した核酸プローブを合成した。

#### 【0169】

合成物の精製：得られた合成物を乾固し乾固物を得た。それを0.5M  $\text{NaHCO}_3$ / $\text{Na}_2\text{CO}_3$ 緩衝液 (pH 9.0) に溶解した。当該溶解物をNAP-25カラム (ファルマシア社製) でゲルろ過を行い、未反応物を除去した。更に逆相HPLC (B gradient: 15~65%、25分間) を以下の条件で行った。そして、溶出するメインピークを分取した。分取した画分を凍結乾燥して、最初のオリゴヌクレオチド原料2mMより23%の収率で核酸プローブを得た。

【0170】

尚、上記の逆相クロマトグラフィーの条件は次の通りである：

溶出溶剤A：0.05N TEAA 5%  $\text{CH}_3\text{CN}$

溶出溶剤B (グラジエント (gradient) 用)：0.05N TEAA  
40%  $\text{CH}_3\text{CN}$

カラム：CAPCEL PAK C18； 6×250mm

溶出速度：1.0ml/min

温度：40℃

検出：254nm

## Example 9

Using a 200-mL Erlenmeyer flask which had been sterilized and which contained sterilized nutrient broth (NB) (50 mL; product of Difco; composition: NB, 0.08 g/100 mL), *Escherichia coli* JM109 was cultured overnight at 37°C under shaking. To the culture, an equivalent amount of 99.7% ethanol was then added. A 2-mL aliquot of the ethanol-added culture was centrifuged in a 2.0-mL Eppendorf centrifuge tube, whereby cells were obtained. The cells were washed once with 30 mM phosphate buffer (sodium salt) (100  $\mu$ L; pH 7.2). The cells were suspended in the phosphate buffer (100  $\mu$ L) which contained 130 mM NaCl. The suspension was ultrasonicated for 40 minutes under ice cooling (output: 33 W, oscillating frequency: 20 kHz, oscillation method: 0.5-second oscillation, followed by a 0.5-second pause), whereby a homogenate was prepared.

After the homogenate was centrifuged, the supernatant was collected and was then transferred into a cell of a fluorimeter. The cell with the supernatant placed therein was controlled at 36°C. A solution of the above-described nucleic acid probe, said solution having had been controlled to 36°C beforehand, was added to the supernatant to give a final concentration of 5 nM. While controlling at 36°C, *E. coli* 16S rRNA and the nucleic acid probe were hybridized for 90 minutes. Intensity of fluorescence emission from the fluorescent dye was then measured by the fluorimeter.

As the intensity of fluorescence emission from the fluorescent dye before the hybridization, a value measured by using 30 mM phosphate buffer (sodium salt), which contained 130 mM NaCl, (pH: 7.2) instead of the above-described supernatant was adopted. Intensity of fluorescence emission was measured by changing the ratio of the amount of the nucleic probe to the amount of the supernatant (exciting light: 503 nm; measured fluorescence color: 512 nm). The results are shown in FIG. 7. As is appreciated from FIG. 7, the intensity of fluorescence emission from the fluorescent dye decreased as the ratio of the amount of the supernatant increased. Namely, it is understood that in the present invention, the magnitude of a decrease in fluorescence emission from a fluorescent dye becomes greater in proportion to the amount of a target nucleic acid to which a nucleic acid probe hybridizes.

#### Example 10

##### Preparation of nucleic acid probe

An oligonucleotide, which was to be hybridized to 23S rRNA of *Escherichia coli* JM109, had a base sequence of (5')CCCACATCGTTTTGTCTGGG(3') and contained  $-(CH_2)_7-NH_2$  bonded to the OH group on the carbon atom at the 3' position of the 5' end nucleotide of the oligonucleotide, was purchased from Midland Certified Reagent Company, U.S.A. as in Example 8. From Molecular Probes, Inc., "FluoroReporter Kit F-6082" (trade name) was also purchased as in Example 8, which contained not

only "BODIPY FL" propionic acid succinimidyl ester but also a reagent for conjugating the compound to the amine derivative of the oligonucleotide. The kit was caused to act on the above-purchased oligonucleotide, whereby a nucleic acid probe  
5 labeled with "BODIPY FL" was synthesized. The synthesized product so obtained was purified as in Example 8, whereby the nucleic acid probe labeled with "BODIPY FL" was obtained with a yield of 25% as calculated relative to 2 mM of the starting oligonucleotide.

#### 10 Example 11

With *Escherichia coli* JM109 cells obtained in Example 9, cells of *Pseudomonas paucimobilis* (now called "*Sphingomonas paucimobilis*) 421Y (FERM P-5122), said cells having have been obtained using the same culture medium and cultivation  
15 conditions as in Example 9, were mixed at the same concentration as *Escherichia coli* JM109 in terms of OD660 value, whereby a co-cultivation system of the microorganisms was prepared. From the resulting mixed system in which the cell concentration of *Escherichia coli* JM109 was the same as that in Example 9,  
20 a homogenate was prepared in the same manner as in Example 9. An experiment was conducted in a similar manner as in Example 9 except that the nucleic acid probe prepared in Example 10 was used, 543 nm exciting light was used, and 569 nm fluorescence was measured. The results were similar to those obtained in  
25 Example 9.

## Example 12

The base selectivity of a target nucleic acid in the quenching phenomenon fluorescence, that is, the base selectivity according to the present invention was investigated. Ten kinds of synthetic target deoxyribooligonucleotides (30 mer; poly a to poly j), which will be described subsequently herein, were prepared by a DNA synthesizer, "ABI394" (trade name; manufactured by Perkin-Elmer Corp.)

Also prepared were the below-described probes according to the present invention, which were labeled with "BODIPY FL" at the 5' ends of deoxyribooligonucleotides corresponding to the above-described synthetic deoxyribooligonucleotides (target genes or target nucleic acids), respectively.

Primer deoxyribooligonucleotides, which corresponded to the above-described synthetic deoxyribooligonucleotides and contained  $-(CH_2)_6-NH_2$  bonded to the phosphate groups at the 5' ends of the primer deoxyribooligonucleotides, were purchased from Midland Certified Reagent Company. From Molecular Probes, Inc., "FluoroReporter Kit F-6082" (trade name) was also purchased, which contained not only "BODIPY FL" propionic acid succinimidyl ester but also a reagent for conjugating the compound to the amine derivative of the deoxyribooligonucleotide. The kit was caused to act on the above-purchased primer deoxyribooligonucleotides, whereby invention nucleic acid probes labeled with "BODIPY FL" (probes

a, b, c, d, f, g, h) were synthesized. An investigation was made under the below-described conditions to determine how much the fluorescence emission from the fluorescent dye would decrease when the probes were caused to hybridize to their corresponding synthetic deoxyribooligonucleotides, and the specificity of the invention probes was studied. Fundamentally, purification was conducted in a similar manner as in Example 8.

<u>Name</u>	<u>Target deoxyribooligonucleotide</u>
poly a	5'ATATATATTTTTTTTGTTTTTTTTTTTT3'
poly b	5'ATATATATTTTTTTTGTTTTTTTTTTTT3'
poly c	5'ATATATATTTTTTTTGTTTTTTTTTTTT3'
poly d	5'ATATATATTTTTTTTGTTTTTTTTTTTT3'
poly e	5'ATATATATTTTTTTTGTTTTTTTTTTTT3'
poly f	5'ATATATATTTTTTTTCTTTTTTTTTTTT3'
poly g	5'ATATATATTTTTTTTCTTTTTTTTTTTT3'
poly h	5'ATATATATTTTTTTTCTTTTTTTTTTTT3'
poly i	5'ATATATATTTTTTTTCTTTTTTTTTTTT3'
poly j	5'ATATATATTTTTTTTCTTTTTTTTTTTT3'

<u>Name</u>	<u>Invention probe</u>
Probe a	3'TATATATAAAAAAAAAACAA5'-BODIPY FL/C6
Probe b	3'TATATATAAAAAAAAAACA5'-BODIPY FL/C6
Probe c	3'TATATATAAAAAAAAAAAC5'-BODIPY FL/C6
Probe d	3'TATATATAAAAAAAAAAAA5'-BODIPY FL/C6
Probe f	3'TATATATAAAAAAAAAAGAA5'-BODIPY FL/C6
Probe g	3'TATATATAAAAAAAAAAGA5'-BODIPY FL/C6
Probe h	3'TATATATAAAAAAAAAAG5'-BODIPY FL/C6

## (1) Components of hybridization mixture

Synthetic DNA	320 nM (final concentration)
Nucleic acid probe	80 nM (final concentration)
NaCl	50 nM (final concentration)
MgCl <sub>2</sub>	1 nM (final concentration)
Tris-HCl buffer (pH 7.2)	100 nM (final concentration)
"MiliQ" purified water	1.6992 mL
Final whole volume	2.0000 mL

(2) Hybridization temperature: 51°C

## (3) Measuring conditions

Exciting light:	503 nm
Measured fluorescence color:	512 nm

Table 2

Nucleic acid probe	Target nucleic acid	Decrease in Fluorescence intensity (%)
a	a	-10
b	b	2
c	c	75
d	d	48
d	e	18
f	f	-8
g	g	-2
h	h	70
d	i	-6
d	j	-5

The results are shown in Table 2. As is appreciated from Table 2, it is preferred to design the base sequence of a nucleic acid probe labeled with a fluorescent dye such that, when the nucleic acid probe hybridizes to a target DNA (deoxyribooligonucleotide), at least one G (guanine) base exists in the base sequence of the target DNA at a position 1 to 3 bases apart from an end base portion where the probe and the target DNA are hybridized with each other. From Table 2, it is also understood to be desired to design the base sequence of a nucleic acid probe labeled with a fluorescent dye such that,

when the nucleic acid probe is hybridized with a target DNA, base pairs in the nucleic acid hybrid complex form at least one G (guanine) and C (cytosine) pair at the end portion.

### Example 13

5 Target nucleic acids and invention nucleic acid probes of the below-described base sequences were prepared. In a similar manner as in the preceding Example, an investigation was made about effects of the number of G(s) in each target nucleic acid and the number of G(s) in its corresponding invention nucleic acid probe.

<u>Name</u>	<u>Target deoxyribooligonucleotide</u>
poly k	5'TATATATATATTTTTGGGGG3'
poly l	5'TATATATATATTTTTGGGG3'
poly m	5'TATATATATTTTTTTTGGG3'
15 poly n	5'TATATATATTTTTTTTGG3'
poly o	5'TATATATATTTTTTTTGG3'
poly p	5'TATATATATTTTTTCCCCC3'
poly q	5'TATATATATTTTTTCCCC3'
poly r	5'TATATATATTTTTTTTCCC3'
20 poly s	5'TATATATATTTTTTTTCC3'
poly t	5'TATATATATTTTTTTTTC3'
poly u	5'TATATATATTTTTTTTTT3'

<u>Name</u>	<u>Invention probe</u>
probe k	3' ATATATATATAAAAAACCC5' -BODIPY FL/C6
probe l	3' ATATATATATAAAAAACCC5' -BODIPY FL/C6
probe m	3' ATATATATATAAAAAACCC5' -BODIPY FL/C6
probe n	3' ATATATATATAAAAAAAC5' -BODIPY FL/C6
probe o	3' ATATATATATAAAAAAAC5' -BODIPY FL/C6
probe p	3' ATATATATATAAAAAGGGG5' -BODIPY FL/C6
probe q	3' ATATATATATAAAAAGGGG5' -BODIPY FL/C6
probe r	3' ATATATATATAAAAAGGG5' -BODIPY FL/C6
probe s	3' ATATATATATAAAAAGG5' -BODIPY FL/C6
probe t	3' ATATATATATAAAAAAAG5' -BODIPY FL/C6
probe u	3' ATATATATATAAAAAA5' -BODIPY FL/C6

Table 3

Nucleic acid probe	Target nucleic acid	Decrease in Fluorescence intensity (%)
k	k	93
l	l	92
m	m	94
n	n	92
o	o	87
p	p	61
q	q	68
r	r	69
s	s	75
t	t	73
u	u	2

As is appreciated from Table 3, neither the number of G(s) in a target nucleic acid nor the number of G(s) in an invention probe substantially affects a decrease in fluorescence intensity.

#### Example 14

Target nucleic acids and invention nucleic acid probes of the below-described base sequences were prepared in a similar manner as described above. The invention nucleic acid probes in this Example were each labeled at the 5' end portion of

oligonucleotide with "BODIPY FL/C6". In a similar manner as in the preceding Example, an investigation was made about effects of the kind of bases in each target nucleic acid and the kind of bases in its corresponding invention nucleic acid probe.

<u>Name</u>	<u>Target deoxyribooligonucleotide</u>
poly W	5'CCCCCCTTTTTTTTTTTT3'
poly X	5'GGGGGGAAAAA3'
poly Y	5'TTTTTTCCCCCCCCCCCC3'
poly Z	5'AAAAAAGGGGGGGGGGG3'

<u>Name</u>	<u>Invention probe</u>
probe w	BODIPY FL/C6-5'AAAAAAGGGGGGG3'
probe x	BODIPY FL/C6-5'TTTTTTTTCCCCCCC3'
probe y	BODIPY FL/C6-5'GGGGGGGGAAAAA3'
probe z	BODIPY FL/C6-5'CCCCCCCCCTTTTTT3'

Table 4

Nucleic acid probe	Target nucleic acid	Fluorescence intensity from probe alone (A)	Fluorescence intensity after addition of target nucleic acid (B)	Decrease in fluorescence intensity, % (C)*
W	w	330	380	-15
X	x	440	430	2
Y	y	40	50	25
Z	z	360	30	92

\* Decrease in fluorescence intensity, % (C) =  $\{(A-B)/A\} \times 100$

As is appreciated from Table 4 and the preceding Example, a substantial decrease takes place in fluorescence intensity (i) when an end of an invention probe labeled with a fluorescent dye is composed of C and hybridization of a target nucleic acid forms a G-C pair, or (ii) when an end of an invention probe labeled with a fluorescent dye is composed of a base other than C and at least one G exists on a side closer to the 3' end of a target nucleic acid than a base pair formed of a base at a location where the invention probe is labeled with the fluorescent dye and a base of the target nucleic acid.

#### Example 15

Concerning the kinds of dyes usable for labeling nucleic acid probes of the present invention, an investigation was made in a similar manner as in the preceding Examples. As an invention probe, the probe z of Example 14 was used. As a target nucleic acid, on the other hand, the oligonucleotide z of Example 14 was employed.

The results are shown in Table 5. As is readily envisaged from this table, illustrative fluorescent dyes suitable for use in the present invention can include FITC, "BODIPY FL", "BODIPY FL/C3", "BODIPY FC/C6", 6-joe, and TMR.

Table 5

Fluorescent dye	Decrease in fluorescence intensity (%)
FITC	90
"BODIPY FL"	95
"BODIPY FL/C3"	98
"BODIPY FL/C6"	97
6-joe	75
TMR	93

Incidentally, the decreases (%) in fluorescence intensity were calculated in a similar manner as in Example 14. Example 16 [Experiment on effects of heat treatment of target nucleic acid (16S rRNA)]

Preparation of invention nucleic acid probe

An oligonucleotide was purchased from Midland Certified Reagent Company, U.S.A. as in Example 8. The oligonucleotide had a base sequence of (5')CATCCCCACC TTCCT CCGAG TTGACCCCGG CAGTC(3') (35 base pairs) hybridizable specifically to the 16S rRNA base sequence of KYM-7 strain, said base sequence being equivalent to the base sequence ranging from the 1156<sup>th</sup> base to the 1190<sup>th</sup> base of the 16S rRNA of *Escherichia coli* JM109, contained deoxyribonucleotides at the 1<sup>st</sup> to 16<sup>th</sup> bases and the 25<sup>th</sup> to 35<sup>th</sup> bases, respectively, and a methyl-modified ribooligonucleotide at the 17<sup>th</sup> to 24<sup>th</sup> bases, said methyl-modified ribooligonucleotide being modified with a methyl group

(modified with an ether bond) at the OH group on the carbon atom at the 2' position, and was modified with  $-(CH_2)_7-NH_2$  at the OH group of the phosphate group at the 5' end. On the other hand, 2-O-Me-oligonucleotide for use in the 2-O-Me probe (a probe formed of a 2-O-Me-oligonucleotide will be simply called "2-O-Me probe") was obtained from GENSET SA, Paris, France by relying upon their custom DNA synthesis services.

From Molecular Probes, Inc., "FluoroReporter Kit F-6082" (trade name) was also purchased, which contained not only "BODIPY FL/C6" propionic acid succinimidyl ester but also a reagent for conjugating the compound to the amine derivative of the oligonucleotide. The kit was caused to act on the above oligonucleotide, whereby a nucleic acid probe labeled with "BODIPY FL/C6" was synthesized. The synthesized product so obtained was purified as in Example 8, whereby the nucleic acid probe according to the present invention labeled with "BODIPY FL/C6" was obtained with a yield of 23% as calculated relative to 2 mM of the starting oligonucleotide. This probe was named "35-nucleotides-chained 2-O-Me probe".

Using a DNA synthesizer, an oligoribonucleotide having a base sequence of (5') TCCTTTGAGT TCCCGGCCGG A (3') was synthesized as in the above to provide it as a forward-type hepter probe. On the other hand, an oligoriboxynucleotide having a base sequence of (5') CCCTGGTCGT AAGGGCCATG ATGACTTGAC GT (3') was synthesized by using a DNA synthesizer, in a similar

manner as described above to provide it as a backward-type, in other words, reverse-type helper probe.

The 35-nucleotides-chained 2-O-Me probe, the forward-type helper probe and the reverse-type helper probe were dissolved in buffer of the below-described composition such that their concentrations reached 25 nM, respectively, and the solution so obtained was heated at 75°C (probe solution).

The above-described 16S rRNA was subjected to heat treatment at 95°C for 5 minutes, and was then added to the probe solution which had been maintained under the below-described reaction conditions. By a fluorescence measuring instrument "Perkin-Elmer LS-50B" (trade name), the intensity of fluorescence was measured. The results are shown in FIG. 8. Incidentally, data obtained by using 16S rRNA which was not subjected to the above-described heat treatment are plotted as a control. It is understood from FIG. 8 that substantial decreases in fluorescence intensity took place in the experimental group subjected to heat treatment. These results indicate that heat treatment of 16S rRNA at 95°C induces stronger hybridization with the probe according to the present invention.

Reaction conditions:

16S rRNA:	10.0 nm
Probe:	25 nM, each
Buffer:	100 mM succinic acid, 125 mM

75°C

75°C

実施例 17 (2'-O-Meオリゴヌクレオチド及びヘルパープローブのハイブリダイゼーション効率向上への寄与の実験)

前記の16SrRNAにハイブリダイズする下記の各種の本発明の核酸プローブ及び各種のヘルパープローブを前記実施例 16と同様にして調製した。また、2'-O-Meプローブに使用する2'-O-Meオリゴヌクレオチドは、すべてGENSET株式会社(フランス)に合成を委託し、得たものである。そして下記の条件にて、本発明の2'-O-Meプローブの効果、当該プローブの塩基鎖の長さの影響、及びヘルパープローブの効果について、下記の図9A、B、C、及びDの実験系で前記実施例 16と同様にして検討した。その結果を図9に示した。

図から、本発明の2'-O-Meプローブがハイブリダイゼーション効率に寄与していることが分かる。また、2'-O-Meプローブの塩基鎖が短い場合にヘルパープローブがハイブリダイゼーション効率を高めるのに役立っている。

## 【0201】

- 1) 35塩基鎖2-o-Meプローブ：前記実施例16と同じプローブ、
- 2) 35塩基鎖DNAプローブ：前記1)の35塩基鎖2-o-Meプローブと同じ塩基配列であるが、オリゴヌクレオチドがデオキシリボースで構成されているプローブ、
- 3) 17塩基鎖2-O-Meプローブ：前記1)の35塩基鎖2-O-Meプローブと同じ塩基配列であるが、5'末端から8塩基分、3'末端から10塩基分のヌクレオチドを削除したプローブ、

- 4) 17塩基鎖DNAプローブ：前記2)の33塩基鎖DNAプローブと同じ塩基配列であるが、3'末端から16塩基分のヌクレオチドを削除したプローブ、

## 【0202】

- 5) フォワード型2-o-Meヘルパープローブ：前記実施例16のフォワード型ヘルパープローブの中央8塩基分（5'末端から数えて9塩基～16塩基分）のリボースの2'位炭素のOH基を、メチル基で修飾（エーテル結合）したヘルパープローブ、

- 6) リバース型2-O-Meヘルパープローブ：前記実施例16のリバース型ヘルパープローブの中央8塩基分（5'末端から数えて9塩基～16塩基分）のリボースの2'位炭素のOH基を、メチル基で修飾（エーテル結合）したヘルパープローブ、

- 7) フォワード型DNAヘルパープローブ：前記実施例16のフォワード型ヘルパープローブの塩基配列と同じ塩基配列であるが、オリゴヌクレオチドがデオキシリボヌクレオチドで構成されているヘルパープローブ、

- 8) リバース型DNAヘルパープローブ：前記 前記実施例16のリバース型ヘルパープローブの塩基配列と同じ塩基配列であるが、オリゴヌクレオチドがデオキシリボヌクレオチドで構成されているヘルパープローブ、

- 9) 35塩基オリゴリボヌクレオチド：(5')CATCCCCACC TTCCTCCGAG TTGACCCCGG CAGTC(3')なる塩基配列を有するオリゴリボヌクレオチド、

- 10) 17塩基鎖オリゴリボヌクレオチド：(5')OCTTCCTCCG AGTTGAC(3')

なる塩基配列を有するオリゴリボヌクレオチド。

### 【0203】

反応条件：

16S rRNA： 10nM

プローブ： 25nM

ヘルパープローブ： 1 $\mu$ M

緩衝液： 100mM コハク酸、125mM 水酸化リチウム、  
8.5% リチウムドデシルサルファイト、pH 5.1

反応温度：

- ・35塩基鎖2-0-Meプローブ： 75℃
- ・17塩基鎖2-0-Meプローブ： 70℃
- ・35塩基鎖DNAプローブ： 75℃
- ・17塩基鎖オリゴリボヌクレオチドDNAプローブ： 60℃

### 【0204】

実験系、図9A：

HP(M)+：16S rRNA、35塩基鎖DNAプローブ、フォワード型2-0-Me ヘルパープローブ、リバーズ型2-0-Meヘルパープローブ、

HP(D)+：16S rRNA、35塩基鎖DNAプローブ、フォワードDNA ヘルパープローブ、リバーズ型DNAヘルパープローブ、

HP-：16S rRNA、35塩基鎖DNAプローブ、

Ref. (対照)：35塩基鎖DNAオリゴリボヌクレオチド、35塩基鎖

## 【0205】

実験系、図9B：

HP(M)+：16SrRNA、35塩基鎖2-0-Meプローブ、フォワード型2-0-Meヘルパープローブ、リバーstype2-0-Meヘルパープローブ、

HP(D)+：16SrRNA、35塩基鎖2-0-Meプローブ、フォワードDNAヘルパープローブ、リバーstypeDNAヘルパープローブ、

HP-：16SrRNA、35塩基鎖2-0-Meプローブ、

Ref. (対照)：35塩基鎖DNAオリゴヌクレオチド、35塩基鎖2-0-Meプローブ。

## 【0206】

実験系、図9C：

HP+(M)：16SrRNA、17塩基鎖DNAプローブ、フォワード型2-0-Meヘルパープローブ、リバーstype2-0-Meヘルパープローブ、

HP+(D)：16SrRNA、17塩基鎖DNAプローブ、フォワード型DNAヘルパープローブ、リバーstypeDNAヘルパープローブ、

HP-：16SrRNA、17塩基鎖DNAプローブ、

Ref. (対照)：17塩基鎖DNAオリゴヌクレオチド、17塩基鎖

## 【0207】

実験系、図9D：

HP+(M)：16S rRNA、17塩基鎖2-0-Meプローブ、フォワード型2-0-Meヘルパープローブ、リバース型2-0-Meヘルパープローブ、

HP+(D)：16S rRNA、17塩基鎖2-0-Meプローブ、フォワード型DNAヘルパープローブ、リバース型DNAヘルパープローブ、

HP-：16S rRNA、17塩基鎖2-0-Meプローブ、

Ref. (対照)：17塩基鎖DNAオリゴヌクレオチド、17塩基鎖2-0-Meプローブ。

### 【0208】

実施例18 (rRNA測定のための検量線の作成)

前記rRNAを、0.1～10nMの範囲のさまざまな濃度において、95℃で5分間加熱後、得られた核酸溶液を予め下記反応条件においた反応液に添加し、1000秒後、蛍光強度の減少をパーキンエルマーLS-50Bを使用して測定した。その結果を図10に示した。図から検量線は0.1～10nMにおいて直線性を示すことが分かる。なお、下記の35塩基鎖2-o-Meプローブは実施例16と同じプローブである。

反応条件：

35塩基鎖2-0-Meプローブ  $\sqrt{\alpha$  溶液 1.0～25nM

緩衝液：100mM コハク酸、125mM 水酸化リチウム、

8.5% リチウムドデシルサルファイト、pH 5.1

反応温度：75℃

### 【0209】

実施例19 (FISH方法)

セルロモナス (Cellulomonas) sp. KYM-7 (FERM P-11339) 及びアグロバクテリウム (Agrobacterium) sp. KYM-8 (FERM P-16806) の各々の rRNA にハイブリダイズする下記の本発明の35又は36塩基鎖オリゴデオキシリボヌクレオチド 2-o-Me プローブを前記と同様にして調製した。各プローブの塩基配列は下記の通りであ

る。

### 【0210】

セルロモナス sp. KYM-7の rRNA 測定のための 35塩基鎖オリゴデオキシリボヌクレオチド2-o-Meプローブ：

(5')CATCCCCACC TTCCTCCGAG TTGACCCCGG CAGTC(3') (アンダーライン部分がメチル基で修飾されている。)

アグロバクテリウム sp. KYM-8の rRNA 測定のための 36塩基鎖オリゴデオキシリボヌクレオチド2-o-Meプローブ：

(5')CATCCCCACC TTCCTCTCGG CTTATCACCG GCAGTC(3') (アンダーライン部分がメチル基で修飾されている。)

### 【0211】

セルロモナス sp. KYM-7及びアグロバクテリウム sp. KYM-8を下記の培地組成の培地で下記の培養条件で混合培養し、培養時間毎に培養物を採取した。それから、rRNAをRNeasy Maxikit(QIAGEN社)を用いて調製した。当該rRNAを95℃で5分加熱後、予め反応条件においた反応液に添加し、70℃、1000秒間反応させた後、蛍光強度をパーキンエルマーLS-50Bを使用して測定した。その結果を図11に示した。尚、全rRNAはリボグリーン(RiboGreen) total RNA Quantification Kit (会社名：モレキュラープローブ(molecular probes)、所在地名：Eugene, Oregon, USA)を用いて測定した。

図から分かるように、各菌株のrRNAの動態は全rRNAの動態と一致した。また、各菌株のrRNAの合計量は全rRNAと一致した。このことは、本発明方法はFISH方法において有効な方法になることを示している。

### 【0212】

・培地組成(g/l)：デンプン，10.0；アスパラギン酸，0.1； $K_2HPO_4$ ，5.0； $KH_2PO_4$ ，2.0； $MgSO_4 \cdot 7H_2O$ ，0.2；NaCl，0.1； $(NH_4)_2SO_4$ ；0.1。

・培地100mlを500ml容のコニカルフラスコに分注し、該フラスコを120℃で10分間、オートクレーブ釜を用いて殺菌した。

・培養条件：前記の菌株を斜面培地で予め培養した。該斜面培地より1白金耳の菌体を取り、前記の殺菌したコニカルフラスコの培地に接種した。30℃、

150rpmで攪拌培養した。

### 【0213】

反応条件：

35塩基鎖オリゴデオキシリボヌクレオチド2-o-Meプローブ： 1.0～  
10nM

緩衝液： 100mM コハク酸、125mM 水酸化リチウム、  
8.5% リチウムドデシルサルファイト、pH 5.1

反応温度： 75℃

### 【0214】

#### 実施例20（鎖内修飾の蛍光消光プローブ実施例）

下記のような塩基配列の標的核酸と本発明の核酸プローブを調製した。  
プローブa), b) 共にAmino-Modifier C6 dC (Glen Research社製) を用いてアミノリンカーをプローブ配列内に導入した後、このアミノリンカーにBODIPY FLラベルしたものを用いた。上記以外の合成法は実施例8と同様である。  
よってプローブa) は、5' 末端のリン酸基ではなく、5' 末端のC塩基に蛍光修飾している。BODIPY FL修飾法、精製方法等は、前記と同様である。

プローブa) 5' C(-BODIPY FL)TTTTTTTCCCCCCCC 3'

プローブb) 5' TTTC(-BODIPY FL)TTTTTTCCCCCCCC 3'

プローブa) の標的核酸c) 5' GGGGGGGGAAAAAAAAAG 3'

プローブb) の標的核酸d) 5' GGGGGGGGAAAAAGAAA 3'

### 【0214】

#### <実験方法>

実施例9と同様の方法で実験を行った。

#### <実験結果>

下の表から明らかなように、プローブa)、プローブb) 共に標的核酸にハイブリダイズしたときに蛍光強度が減少することが明らかとなった。 プローブb) の結果より、5' 末端あるいは3' 末端以外のDNA鎖内のシトシン塩基に蛍光修飾することでも、蛍光消光プローブとして機能することが明らかとなっ

た。また、プローブ a) の結果より、末端のシトシンであっても 5' 末端のリン酸基または 3' 末端の OH 基以外の部位に蛍光修飾することで、蛍光消光プローブが得られることも明らかとなった。

## 【0216】

表6 実施例20の結果

	ハイブリダイゼーション前蛍光強度	ハイブリダイゼーション後蛍光強度	蛍光消光率 (%)
プローブ a) + 標的核酸 c)	410	75	81.7
プローブ b) + 標的核酸 d)	380	82	78.4

## 【0217】

以下実施例 21 に、標的核酸の多型及び変異を解析若しくは測定する方法を記す。

## 実施例 21

下記に示した塩基配列をもつ 4 種類のオリゴヌクレオチドを前記実施例 12 の DNA 合成機を用いて合成した。また、前記実施例 12 と同様にして、下記の塩基配列の本発明の核酸プローブを合成した。該プローブと各々のオリゴヌクレオチドを溶液中でハイブリダイズさせた後、蛍光強度の変化から 1 塩基置換の評価ができるかどうか検討した。本発明の核酸プローブの塩基配列は、標的オリゴヌクレオチドのうちのいずれかの 3' 末端に G が存在する場合に、そのオリゴヌクレオチドの塩基配列に 100% マッチするように設計されている。ハイブリダイゼーション温度は、プローブと標的オリゴヌクレオチドとの間の全塩基対 (base-pairs) が 100% ハイブリダイズできる 40℃ に設定した。プローブ及び標的オリゴヌクレオチドの濃度、緩衝液の濃度、蛍光測定装置、蛍光測定条件、実験操作などは、前記実施例 12 と同様である。

## 【0218】

本発明のプロープ：3'TTTTTTTTGGGGGGGC5'BODIPY FL/C6

標的オリゴヌクレオチドNo. 1：5'AAAAAAACCCCCCA3'

標的オリゴヌクレオチドNo. 2：5'AAAAAAACCCCCCCC3'

標的オリゴヌクレオチドNo. 3：5'AAAAAAACCCCCCCI3'

(I:hypoxanthine)

標的オリゴヌクレオチドNo. 4：5'AAAAAAACCCCCCG3'

### 【0219】

その結果を表7に示した。表から、標的オリゴヌクレオチドNo. 1～3においては、蛍光強度に変化は観察されなかったが、標的オリゴヌクレオチドNo. 4においては84%の減少が観察された。

### 【0220】

表7

標的オリゴヌクレオチド	初期蛍光強度 (A)	ハイブリダイゼーション後の蛍光強度 (B)	(A-B) / B
No. 1	340	350	-0.03
No. 2	332	328	0.01
No. 3	343	336	0.02
No. 4	345	52	0.84

### 【0221】

本発明において、標的核酸（例えば上記標的オリゴヌクレオチドNo. 1～4）の多型及び／又は変異を解析若しくは測定する方法により得られるデータ（例えば表7のカラムA及びBのデータ）を解析する方法において、標的核酸が本発明の核酸プローブ（上記の核酸プローブ）とハイブリダイズしたときの反応系の蛍光強度値を、前記のハイブリダイズしていないときの反応系の蛍光強度値により補正演算処理するとは、表4の(A-B) / Bの計算をいう。

以上の結果より、標的核酸が2本鎖の場合、G→A、G←A、C→T、C←T、G→C、G←Cの置換を検出できることが明らかになった。

## 実施例 22

図12に本発明のDNAチップのモデルの一例を図示した。先ず、実施例21で調製した本発明のプロープである3' TTTTTTTTGGGGGGGC5' BODIPY FL/C6の3'末端のリボースの3'位炭素のOH基にアミノ基を導入して調製した修飾プロープ、また、スライドガラスを反応基としてエポキシ基を有するシランカップリン剤でスライドガラスの表面を処理した表面処理済スライドガラスを用意した。上記の修飾プロープを含む溶液をDNAチップ作成装置GMS™417ARRAYER(TAKARA)で該表面処理済スライドガラス上にスポットした。そうすると、3'末端で上記修飾プロープがガラス面に結合した。該スライドガラスを密閉容器内に4時間位おき反応を完結させた。そして該スライドガラスを0.2% SDS溶液、水に1分程度交互に2回ずつ漬けた。更にホウ素溶液(水300mlにNaBH<sub>4</sub>1.0gを溶かしたもの。)に5分位つけた。95℃の水に2分つけてから、素早く0.2% SDS溶液、水に1分程度交互に2回ずつ漬けて試薬を洗い流した。室温で乾燥した。このようにして本発明のDNAチップを調製した。

## 【0223】

更に、ガラスの下面の修飾プロープの各スポットに対応する位置に図のような微小な温度センサーとヒータを設けることにより、本発明のDNAチップに高性能を付与することができる。

このDNAチップを用いて標的核酸を測定する場合を説明する。該プロープに標的核酸がハイブリダイズしていないとき、又はハイブリダイズしても蛍光色素標識末端でGCペアーを形成しないとき、若しくは当該プロープと標的核酸とがハイブリダイズした末端塩基部分から1ないし3塩基離れて、標的核酸の塩基配列にG(グアニン)又はシトシン(C)が少なくとも1つ存在しないときは、蛍光強度に変化ない。しかし、その反対に、該プロープに標的核酸がハイブリダイズしているとき、又はハイブリダイズしても蛍光色素標識末端でGCペアーを形成しているとき、若しくは当該プロープと標的核酸とがハイブリダイズした末端塩基部分から1ないし3塩基離れて、標的核酸の塩基配列にG(グアニン)又はシトシン(C)が少なくとも1つ存在するときは蛍光強度が減少する。この蛍光

強度はDNAチップ解析装置GMS™418アレーsscanner (Array Scanner) (TAKARA) を使用して測定できる。

#### 【0224】

実施例23：本発明のDNAチップを用いた一塩基多型 (SNPs) の検出実験

I) 標的核酸の調製：(5')AAACGATGTG GGAAGGCCCA GACAGCCAGG ATGTTGGCTT AGAAGCAGCC(3')の塩基配列をもつオリゴデオキシリボヌクレオチドを、DNA合成機ABI394(Perkin Elmer社製、米国)を用いて合成し、標的核酸とした。

I I) 核酸プローブの調製：標的核酸の5'末端から15塩基の配列(アンダーライン部)にハイブリダイズする塩基配列をもつ、下記の6個のオリゴデオキシリボヌクレオチドを、DNA合成機ABI394(Perkin Elmer社製、米国)を用いて合成した。そして、3'-Amino-Modifier C7 CPG(グレンリサーチ、カタログ番号20-2957)を用いて、3'末端のデオキシリボースの3'位のOH基をアミノ化した。更に5'末端のリン酸基を実施例12と同様な方法でBODIPY FLで標識した。

#### 【0225】

- 1) プローブ100 (100%マッチ)：(5')CCTTCCCACA TCGTTT(3')、
- 2) プローブ-T (1塩基ミスマッチ)：(5')CCTTCCCATA TCGTTT(3')、
- 3) プローブ-A (1塩基ミスマッチ)：(5')CCTTCCCAAA TCGTTT(3')、
- 4) プローブ-G (1塩基ミスマッチ)：(5')CCTTCCCAGA TCGTTT(3')、
- 5) プローブ-TG (2塩基ミスマッチ)：(5')CCTTCCCTGA TCGTTT(3')、
- 6) プローブ-TGT (3塩基ミスマッチ)：(5')CCTTCCCTGT TCGTTT(3')、

#### 【0226】

#### III) DNAチップの調製

全てのDNAプローブを、0.1M MES(2-Morpholinoethanesulfonic acid)緩衝液(pH, 6.5)に溶解して、500nM濃度の溶液にした。DNAマイクロアレイ装置(DNAマイクロアレイNo. 439702、32ピン型、およびDNAスライドインデックスNo. 439701からなる手動式のチップアレイである。Greiner社製)を用いて、DNAチップ用スライドガラス(Black silylated slides、Greiner社製)の上に、前記プローブ溶液をスポテイング(spotting)した。スポット終了

後、湿性チャンバー内で60分間室温で反応させ、プローブをスライドガラス上に固定化した。その後、50mMのTE緩衝液(pH:7.2)にて洗浄した。なお、各プローブ溶液につき、4スポットずつスポットングした。固定化後スライドガラスを、0.1%SDS(sodium dodecylsulfate)溶液にて1回洗浄後、蒸留水にて2回洗浄し、水酸化ホウ酸ナトリウム溶液(2.5mg-NaBH<sub>4</sub>/ml-25%エタノール溶液)内に5分間浸した。その後、3分間95℃に加温した湯浴中に浸した後、乾燥させた。

本発明のDNAチップの模式図を図12に示した。スライドガラス上に固定化された本発明のプローブは、標的核酸にハイブリダイズしないときはBODIPY FLは発色しているが、ハイブリダイズしているときは発色が、ハイブリダイズしないときのものよりも少ない。すなわち減少する。スライドガラスはマイクロヒーターで加熱されるようになっている(本発明では、下記に示すように顕微鏡用透明加熱板(MP-10MH-PG、(株式会社)北里サプライ)で行われた。))。

#### 【0227】

##### IV)SNPsの検出測定

100μM濃度の標的核酸溶液{50mMのTE緩衝液(pH:7.2)使用}を上記のごとくに調製したDNAチップの上にのせた。カバーガラスで覆い、標的核酸が漏れないようにマニキュアにてカバーガラスをシールした。検出測定のための装置類の概略は図13に示した。先ず、オリンパス正立焦点レーザー顕微鏡(AX80型)の試料台に顕微鏡用透明加熱板(MP-10MH-PG、(株式会社)北里サプライ)をのせた。当該板上に前記に調製した本発明のDNAチップを置き、95℃から33℃まで3℃刻みで変化させ、30分かけて反応させた。各スポットの反応過程の蛍光強度変化を、冷却CCDカメラ(C4880-40型、浜松フォトニクス社)にて画像取り込み形式で測定した。

取り込み画像を画像解析装置(画像解析ソフト(TPlab spectrum; Signal Analytics社, Virginia)がインストールされたパソコン(NEC))にて解析し、各スポットの輝度を算出し、温度と輝度の関係を求めた。

#### 【0228】

実験結果を図14に示した。図から全てのプローブで蛍光強度が減少している

ことが分かる。従って、本発明の方法により、本発明のプロープと標的核酸との解離曲線を簡便にモニタリングすることができる。また、標的核酸と100%マッチするプロープ100と一塩基ミスマッチするプロープとの $T_m$ 値の差は10℃以上あるので、解離曲線から両者を容易に識別することができる。すなわち本発明のDNAチップを使用することによりSNPsの解析が容易にできることがわかる。

#### 【0229】

以下、実施例24～27に本発明のPCR方法を記す。

#### 実施例24

大腸菌のゲノムDNAにおける16SrRNA遺伝子を標的核酸として、当該核酸の増幅のための(BODIPY FL/C6で標識した)プライマー(本発明の核酸プロープ)を調製した。

#### 【0230】

プライマー1(Eu800R:リバース型)の調製:(5')CATCGTTTAC GCGTGGAC(3')の塩基配列をもつオリゴデオキシリボヌクレオチドを、DNA合成機ABI394(Perkin Elmer社製、米国)を用いて合成し、更に当該オリゴデオキシリボヌクレオチドの5'末端のリン酸基をホスファターゼ処理してシトシンとし、そのシトシンの5'位の炭素OH基に、 $-(CH_2)_9-NH_2$ を結合したオリゴヌクレオチドを、ミドランド・サーティファイド・レージンド・カンパニー社(米国)から購入した。更に、モレキュラープロープ社からフロオ・リポーターキット(FluoReporter Kits)F-8082(ボデピーFL/C6のプロピオン酸サクシニミジルエステル(BODIPY FL propionic acid succinimidyl ester)の他に、当該化合物をオリゴヌクレオチドのアミン誘導体に結合させる試薬を含有するキット)を購入した。前記購入したオリゴヌクレオチドに当該キットを作用させて、本発明のボデピーFL/C6で標識したプライマー1を合成した。

#### 【0231】

合成物の精製:得られた合成物を乾固し乾固物を得た。それを0.5M  $Na_2CO_3/NaHCO_3$ 緩衝液(pH9.0)に溶解した。当該溶解物をNAP-25カラム(ファルマシア社製)でゲルろ過を行い、未反応物を除去した。更に逆相HPLC(B

gradient: 15 ~ 65%, 25分間) を以下の条件で行った。そして、溶出するメインピークを分取した。分取した画分を凍結乾燥して本発明のプライマー1を、最初のオリゴヌクレオチド原料2 mMより50%の収率で得た。

#### 【0232】

尚、上記の逆相クロマトグラフィーの条件は次の通りである：

溶出溶剤A：0.05N TEAA 5%CH<sub>3</sub>CN

溶出溶剤B（グラジエント(gradient)用）：0.05N TEAA  
40%CH<sub>3</sub>CN

カラム：CAPCEL PAK C18 ; 6×250mm

溶出速度：1.0 ml/min

温度：40℃

検出：254 nm

#### 【0233】

##### 実施例25

プライマー2 (Eu500R/forward：フォワード型) の調製：(5')CCAGCAGCCGCGTAATAC(3')の塩基配列をもつオリゴデオキシリボヌクレオチドの5'末端に蛍光色素(BODIPY FL/C6)で標識したプライマー2を実施例2と同様にして収率50%で調製した。

#### 【0234】

##### 実施例26

殺菌したニュートリエントブロス(NB) (Difco社製) 液体培地5 ml (組成：NB、0.08g/100ml) を含有する試験管を用いて、大腸菌JM109株を37℃で一晩振盪培養した。培養液1.5mlを1.5ml容量の遠心チューブで遠心分離し、菌体を得た。この菌体から、DNeasy Tissue Kit (キアゲン (QIAGEN) 社、ドイツ国) を用いてゲノムDNAを抽出した。その抽出は本キットのプロトコルに従った。その結果、17 ng/μlのDNA溶液を得た。

#### 【0235】

##### 実施例27

上記の大腸菌のゲノムDNA、プライマー1及び／又はプライマー2を使用して、ロシュ・ダイアグノスティックス株式会社発売のライトサイクラー™システム (LightCycler™ System) を用いて常法通りにPCR反応を行った。操作は当該システム機器の手順書に従った。

また、上記システムにおいてPCRは、当該手順書に記されている核酸プローブ (FRET現象を利用する二個のプローブ) と通常のプライマー (蛍光色素で標識されていない通常のプライマー) の代りに本発明プライマー1及び／又は2を用いる以外は当該手順書通りに行った。

#### 【0236】

PCRは次のコンポーネントのハイブリダイゼーション溶液中で行った。

大腸菌ゲノムDNA溶液	3.5 $\mu$ l (終濃度0~6 ng / 20 $\mu$ l) (終コピー数0~2.4 $\cdot 10^6$ 個)
プライマー溶液	0.8 $\mu$ l (終濃度0.08 $\mu$ M)
Taq溶液	10.0 $\mu$ l
ミリQ純水	5.7 $\mu$ l
全容量	20.0 $\mu$ l

尚、標的核酸である大腸菌16S rDNAは、図15の説明欄に示される実験区の濃度で、また、プライマーは、同様に図15の説明欄に示される実験区のプライマー1及び／又は2の組合せで実験を行った。

#### 【0237】

また、上記のTaq溶液は次の試薬の混合液である。

Taq 溶液	96.0 $\mu$ l
ミリQ純水	68.2 $\mu$ l
Taq DNA ポリメラーゼ溶液	24.0 $\mu$ l
Taq スタート(start)	3.8 $\mu$ l

### 【0238】

尚、Taq 溶液、Taq DNAポリメラーゼ溶液はロシュ・ダイアグノスティックス株式会社発売のDNAマスターハイブリダイゼーションプローブ (DNA Master Hybridization Probes)キットのものである。特にTaq DNA ポリメラーゼ溶液は10×conc. (赤いキャップ)を10倍に希釈して用いた。また、Taq スタートは、クローンテック社 (USA) より販売されているTaq DNAポリメラーゼ用の抗体で、これを反応液に添加することで70℃までTaq DNAポリメラーゼの活性を抑えることができる。即ち、ホット・スタート (hot start)を行うことができるものである。

### 【0239】

反応条件は次の如くである。

変性 (denaturation)	初期：95℃、120秒
	2回目以降：95℃、10秒
アニーリング (annealing)	57℃、5秒

測定は、ライトサイクラー<sup>TM</sup>システムを用いて行った。その際、該システムにあるF1～3の検出器のうち、F1の検出器を用い、その検出器のゲインは10、励起強度は75に固定した。

### 【0240】

その結果を図15及び16に示した。図15及び16から、蛍光色素の発光の減少が観察される時点のサイクル数と標的核酸の大腸菌16SrDNAのコピー数が比例していることが分かる。尚、図においては、蛍光色素の発光の減少量を

蛍光強度の減少値として表現した。

図17は、サイクル数の関数として、大腸菌16S rDNAのコピー数を表現した大腸菌16S rDNAの検量線を示す。相関係数は0.9973で、極めてよい相関を示した。

以上の結果から分かるように、本発明の定量的PCR方法を用いると標的核酸の当初のコピー数を測定できるようになる。即ち、標的核酸の濃度の測定ができる。

【0241】

#### 実施例28

実施例27においては、本発明のプローブをプライマーとしてPCRを行ったが、本実施例では従来法に用いるFRET現象を利用する二個のプローブの代わりに本発明のプローブを用いて下記の条件で本発明のPCRを行った。

a) 標的核酸：大腸菌の16S-rDNA

b) 使用プライマー：

・フォワードプライマー E8F:(5')AGAGTTTGAT CCTGGCTCAG(3')

・リバースプライマー E1492R:(5')GGTTACCTTG TTACGACTT(3')

c) 使用プローブ：BODIPY FL-(5')CGGGCGGTGT GTAC(3') (但し、3'末端はリン酸化されたもの)

d) 使用PCR測定機器：ライトサイクラー<sup>TM</sup>システム

e) PCRの条件：

変性反応 : 95℃、10秒 (第一回のみ、60秒間、95℃)

アニーリング反応 : 50℃、5秒

核酸伸長反応 : 72℃、70秒

全サイクル数 : 70サイクル

## 【0242】

f) 蛍光測定 (アニーリング反応と変性反応後各サイクルー回ずつ測定された。

g) 反応液の組成:

反応液の全量:  $20\mu\text{l}$

DNA ポリメラーゼの量 (TaKaRa Ex taq): 0.5U

Taq スタート(抗体):  $0.3\mu\text{l}$

プライマーの濃度:  $0.2\mu\text{M}$  (双方とも)

プローブの濃度:  $0.05\mu\text{M}$

MgCl<sub>2</sub> 濃度: 2 mM

BSA(bovine serum albumin)濃度: 0.25 mg/ml

dNTPs濃度: 2.5 mM (各ヌクレオチドについて).

## 【0243】

その結果を図18に示した。図から、蛍光色素の発光の減少が観察される時点のサイクル数と標的核酸の大腸菌16SrDNAのコピー数が比例していることが分かる。

以上の結果から分かるように、本発明の定量的PCR方法を用いると標的核酸の当初のコピー数を測定できるようになる。即ち、標的核酸の測定ができる。

## 【0244】

次に以下の実施例に、上記の本発明の定量的PCR方法を用いて得られるデータを解析する本発明のデータ解析方法について記す。

## 実施例29

ヒトゲノムDNA (ヒトβ-グロビン (globin) (TaKaRaカタログ商品番号 9060) (TaKaRa株式会社製) (以下、ヒトゲノムDNAという。)) を標的核酸として、当該核酸の増幅のためのボデピー FL/C6で標識したプライマーを調製した。

## 【0245】

プライマーKM38+C (リバース型) の調製: (5')CTGGTCTCCT TAAACCTGTC TTG

(3')の塩基配列をもつオリゴデオキシリボヌクレオチドを、DNA合成機ABI394 (Perkin Elmer社製、米国)を用いて合成し、更に当該オリゴデオキシリボヌクレオチドの5'末端のリン酸基をホスファターゼ処理してシトシンとし、そのシトシンの5'位の炭素OH基に、 $-(CH_2)_9-NH_2$ を結合したものを、ミドランド・サーティファイド・レージンド・カンパニー社から購入した。更に、モレキュラープロブ社からリポーターキット(FluoReporter Kit)F-6082 (ボデピーFL/C6のプロピオン酸サクシニミジルエステル (BODIPY FL propionic acid succinimidyl esters)の他に、当該化合物をオリゴヌクレオチドのアミン誘導体に結合させる試薬を含有するキット)を購入した。前記購入したオリゴヌクレオチドに当該キットを作用させて、本発明のボデピーFL/C6で標識したプライマーKM38+Cを合成した。

#### 【0246】

合成物の精製：得られた合成物を乾固し乾固物を得た。それを0.5M  $Na_2CO_3/NaHCO_3$ 緩衝液(pH 9.0)に溶解した。当該溶解物をNAP-25カラム(ファルマシア社製)でゲルろ過を行い、未反応物を除去した。更に逆相HPLC(B gradient:15~85%、25分間)を以下の条件で行った。そして、溶出するメインピークを分取した。分取した画分を凍結乾燥して本発明のプライマーKM38+Cを、最初のオリゴヌクレオチド原料2mMより50%の収率で得た。

#### 【0247】

尚、上記の逆相クロマトグラフィーの条件は次の通りである：

溶出溶剤A：0.05N TEAA 5% $CH_3CN$

溶出溶剤B (グラジエント (gradient) 用)：0.05N  
TEAA 40% $CH_3CN$

カラム：CAPCEL PAK C18;6×250mm

溶出速度：1.0ml/min

温度：40℃

検出：254nm

#### 【0248】

## 実施例 30

プライマー KM29 (フォワード型) の調製: (5') GGT TGG CCAA  
TCT ACT CCA G G (3') の塩基配列をもつオリゴデオキシリボヌ  
クレオチドを実施例 26 と同様に合成した。

【0249】

## 比較実験例 1

本比較実験例は、核酸伸長反応時の蛍光強度値を、熱変性反応時の蛍光強度値を用いて割る演算処理過程(数式(1)の処理)を有しないデータ解析用ソフトウェアの使用に係るものである。

上記のヒトゲノム DNA、プライマー KM38 + C 及びプライマー KM29 を使用して、ライトサイクラー<sup>TM</sup>システムを用いて PCR 反応を行い、各サイクル毎の蛍光強度を測定した。

尚、本比較実験例の PCR は、前記に説明した~~蛍光色素色素~~で標識したプライマーを用いるものであり、蛍光発光の増加でなく、減少を測定する新規なリアルタイム定量的 PCR 方法である。データ解析は当該システムのソフトウェアを用いて行った。本比較実験例の PCR は、当該手順書に記されている核酸プローブ(FRET 現象を利用する二個のプローブ)と通常のプライマー(蛍光色素で標識されていない通常のプライマー)の代りに本発明プライマー KM38 + C 及び KM29 を用いる以外は当該装置の手順書通りに行った。

【0250】

PCR は次のコンポーネントのハイブリダイゼーション溶液中で行った。

ヒトゲノム DNA	1.0 $\mu$ l (最終濃度 1~10000 コピー)
プライマー溶液	4.0 $\mu$ l (最終濃度 0.1 $\mu$ M)
Taq 溶液	10.0 $\mu$ l
ミリ Q 純水	5.0 $\mu$ l
全容量	20.0 $\mu$ l

尚、ヒトゲノム DNA は、図 19 の簡単な説明欄に示される実験区の濃度で実験を行った。MgCl<sub>2</sub> の最終濃度は 2 mM であった。

## 【0251】

また、上記のTaq溶液は次に試薬の混合液である。

Taq 溶液	96.0 $\mu$ l
ミリQ純水	68.2 $\mu$ l
Taq DNA ポリメラーゼ	24.0 $\mu$ l
Taq スタート	3.8 $\mu$ l

## 【0252】

尚、Taq溶液、Taq DNA ポリメラーゼ溶液はロシュ・ダイアグノスティック株式会社発売のDNAマスターハイブリダイゼーションプローブ (DNA Master Hybridization Probes) キットのものである。特にTaq DNA ポリメラーゼ溶液は10×conc. (赤いキャップ) を10倍に希釈して用いた。また、Taqスタートは、クロンテック社 (USA) より販売されているTaq DNA ポリメラーゼ用の抗体で、これを反応液に添加することで70℃までTaq DNAポリメラーゼの活性を抑えることができる。即ち、ホット・スタートを行うことができるものである。

## 【0253】

反応条件は次の如くである。

変性反応初期	: 95℃、60秒
再 (2回目以降の) 変性反応	: 95℃、10秒
アニーリング反応	: 60℃、5秒
DNA伸長反応	: 72℃、17秒

測定は、ライトサイクラー™システムを用いて行った。その際、該システムにあるF1～3の検出器のうち、F1の検出器を用い、その検出器のゲインは10、励起強度は75に固定した。

## 【0254】

前記の如くにPCRを行って、各サイクルの蛍光強度を実測した。その結果を図19に示す。即ち、各コピー数のヒトゲノムDNAについて、各サイクルの変性反応時及び核酸伸長反応時の蛍光強度を測定し、印字したものである。どのサイクルにおいても変性反応時には蛍光強度値は一定であるが、核酸伸長反応時には、25サイクル目当たりから蛍光強度が減少しているのが観察される。そうして、減少はヒトゲノムDNAのコピー数が多い順に起こることが分かる。

#### 【0255】

図19に示すようにヒトゲノムDNAの各コピー数について初期のサイクル数の蛍光強度値が一様でなかった。それで、本比較例で使用するデータ解析方法に以下の過程(b)～(j)を追加した。

(b) 10サイクル目の蛍光強度値を1として各サイクルの蛍光強度値を換算する過程、即ち、下記の〔数式8〕による計算をする過程、

$$C_n = F_n(72) / F_{10}(72) \quad \text{〔数式8〕}$$

ただし、 $C_n$  = 各サイクルにおける蛍光強度値の換算値、 $F_n(72)$  = 各サイクルの72℃の蛍光強度値、 $F_{10}(72)$  = 10サイクル目の72℃における伸長反応後の蛍光強度値。

(c) 前記(b)の過程で得られた各換算値を、サイクル数の関数として、ディスプレイ上に表示及び／又は印字する過程、

#### 【0256】

(d) 前記(b)の過程で得られた各サイクルの換算値から下記の〔数式9〕による蛍光強度の変化率(減少率、消光率)を計算をする過程、

〔数式9〕

$$F_{dn} = 10 \log_{10} \{100 - C_n \times 100\}$$

$$F_{dn} = 21 \log_{10} \{1 - C_n\}$$

ただし、 $F_{dn}$  = 蛍光強度変化率(減少率、消光率)、 $C_n$  = 〔数式8〕で得られた値。

(e) 前記(d)の過程で得られた各換算値を、サイクル数の関数として、ディスプレイ上に表示及び／又は印字する過程、

#### 【0257】

(f) 前記 (d) の過程で処理されたデータを、スレッシュホールド (threshold) としての 0.5 と比較し、その値に達したサイクル数を計数する過程、

(g) 前記 (f) の過程で計数した値を X 軸に、反応開始前のコピー数を Y 軸にプロットしたグラフを作成する過程、

(h) 前記 (g) の過程で作成したグラフをディスプレイ上に表示及び／又は印字する過程、

(i) 前記 (h) の過程で描かれた直線の相関係数又は関係式を計算する過程、

(j) 前記 (i) の過程で計算された相関係数又は関係式をディスプレイ上に表示及び／又は印字する過程。

#### 【0258】

上記のデータ解析用ソフトウェアを用いて、前記図 19 で得られたデータを前記に引き続いて以下のように処理した。

図 20 は、上記 (b) の過程で処理されたデータを印字した (前記 (c) 過程) したものである。即ち、10 サイクル目の蛍光強度値を 1 として各サイクルの蛍光強度を換算し、その換算値を対応するサイクル数に対してプロットしたものである。

図 21 は、前記 (d) の過程で処理したデータを印字した (前記 (e) 過程) ものである。即ち、図 20 の各プロット値から蛍光強度の減少率 (消光率) を計算して、各計算値を各サイクル数に対してプロットしたものである。

#### 【0259】

図 22 は、前記 (f) の過程で処理したデータについて、前記 (g) の過程で作成したグラフを印字した (前記 (h) の過程) ものである。即ち、蛍光強度減少率 = 0.5 をスレッシュホールド (threshold) し、その値に達したサイクル数を X 軸に、ヒトゲノム DNA の反応開始前のコピー数を Y 軸にプロットしたグラフである。このグラフの直線の相関係数 ( $R^2$ ) を前記 (i) の過程で計算し、印字した (前記 (j) の過程) もので、0.9514 であった。このように、この相関係数では正確なコピー数を求めるは無理であった。

#### 【0260】

実施例31（本発明のデータ解析方法を用いてデータ処理がなされた実験例）

PCRは比較実験例1と同様に行った。データ処理は、比較実験例1の（b）の過程の前に下記の（a）の過程をおき、（b）、（d）の過程を以下のように変更する以外は比較実験例1と同様な過程で行った。

（a）各サイクルにおける増幅した核酸が本発明の核酸プローブである（蛍光色素で標識された）核酸プライマーとハイブリダイズしたときの反応系の蛍光強度値（即ち、核酸伸長反応時（72℃）の蛍光強度値）を、核酸ハイブリッド複合体（増幅した核酸が核酸プライマーとハイブリダイズしたもの）が解離したときに測定された反応系の蛍光強度値（即ち、核酸熱変性反応完了時（95℃）の蛍光強度値）で割る補正演算処理過程、即ち、実測の蛍光強度値を次の〔数式1〕で補正した。

$$f_n = f_{hyb, n} / f_{den, n} \quad \text{〔数式1〕}$$

〔式中、 $f_n$  = サイクルの蛍光強度の補正值、 $f_{hyb, n}$  = 各サイクルの72℃の蛍光強度値、 $f_{den, n}$  = 各サイクルの95℃の蛍光強度値〕

得られた値を各サイクル数に対してプロットしたのが図23である。

【0261】

（b）各サイクルにおける〔数式1〕における補正演算処理値を〔数式3〕に代入し、各サイクルにおける各サンプル間の蛍光変化率（減少率又は消光率）を算出する演算処理過程、即ち、下記の〔数式10〕で演算処理する過程、

$$F_n = f_n / f_{25} \quad \text{〔数式10〕}$$

〔式中、 $F_n$  = 各サイクルの演算処理値、 $f_n$  = 〔数式1〕で得られた各サイクルの値、 $f_{25}$  = 〔数式1〕で得られた値で、サイクル数が25回目のもの〕。

〔数式10〕は〔数式3〕において、 $a = 25$ とした場合におけるものである。

【0262】

（d）前記（b）の過程で得られた各サイクルの演算処理値を〔数式6〕による蛍光強度の変化率（減少率又は消光率）の対数値を得るための演算処理に付す過程、即ち、下記の〔数式11〕で演算処理する過程、

$$\log_{10} \{ (1 - F_n) \times 100 \} \quad \text{〔数式11〕}$$

〔式中、 $F_n$  = 〔数式10〕で得られた値〕。

〔数式11〕は〔数式6〕において、 $b = 10$ 、 $A = 100$ とした場合におけるものである。

#### 【0263】

上記の結果を図24及び25に示した。

図24は、前記(a)及び(b)の過程で処理された値をサイクル数に対してプロットし、印字したものである。

図25は、図24で得られた値を前記(d)の過程のように処理して得られた値を、サイクル数に対してプロットし、印字したものである。

#### 【0264】

次に、図25のグラフを基に、前記(f)、(g)、及び(h)の過程で処理した。即ち、図25のグラフを基に比較実験例1と同様に、 $\log_{10}$ (蛍光強度変化率)のスレッシュホールド値として、0.1、0.3、0.5、0.7、0.9、1.2を選び、その値に達したサイクル数をX軸に、ヒトゲノムDNAの反応開始前のコピー数をY軸にプロットし、検量線を描かせた。その結果を図26に示した。これらの検量線について前記(i)及び(j)の過程で処理して求めた相関係数( $R^2$ )は、前記各スレッシュホールド値に対して、各々0.998、0.999、0.9993、0.9985、0.9989、0.9988であった。これらの相関係数から、スレッシュホールド値として0.5(相関係数0.9993)を採用することが望ましいことが認識できた。この相関係数をもつ検量線であれば、未知コピー数の核酸試料について反応開始前のコピー数を精度よく求めることができることが分かる。

#### 【0265】

実施例32(核酸の融解曲線分析及び $T_m$ 値分析の例)

✓ 本発明の新規なPCR法により増幅された核酸について、~~1~~1)低い温度から核酸が完全に変性するまで、温度を徐々に上げるあるいは下げる過程(例えば、  
✓ 50℃から95℃まで)、~~2~~2)前記~~1~~1)過程において、短い時間間隔(例えば、0.2℃~0.5℃の温度上昇に相当する間隔)で蛍光強度を測定する過程、  
✓ ~~3~~3)前記~~2~~2)過程の測定結果を時間の関数としてディスプレイ上に表示す

る過程、即ち、核酸の融解曲線を表示する過程、~~5~~4) 前記~~5~~3) 過程の融解曲線を一次微分する過程、~~5~~5) 前記~~5~~4) 過程の微分値 ( $-dF/dT$ 、 $F$ : 蛍光強度、 $T$ : 時間) をディスプレイ上に表示する過程、~~5~~6) 前記~~5~~5) から得られる微分値から変曲点を求める過程からなるソフトウェアを作成し、前記本発明のデータ解析用ソフトウェアに合体した。当該データ解析用ソフトウェアを記録したコンピューター読み取り可能な記録媒体をインストールした前記ライトサイクラー<sup>TM</sup>システムを用いて本発明の新規リアルタイム定量的PCR反応を行い、核酸融解曲線の分析を行った。本発明においては、蛍光強度は温度が上がることに増加する。

#### 【0266】

実施例31と同じヒトゲノムDNAの1コピーと10コピーについて、実施例29と同様のPCRを行い、前記~~5~~1)、~~5~~2)、~~5~~3)、~~5~~4) 及び~~5~~5) の過程で処理されたデータを印字したものが図27である。1コピーと10コピーの75回目の増幅産物について、本実施例の~~5~~1)、~~5~~2) 及び~~5~~3) の過程で処理した核酸融解曲線の図が図28である。~~5~~4) の過程でこの曲線を微分し、~~5~~5) 及び~~5~~6) の過程で変曲点 ( $T_m$  値) を求めたものが図29である。図29から、1コピーと10コピーの増幅産物の  $T_m$  値が異なる故に、各増幅産物は異なる産物であることが判明した。

#### 【0267】

以下の実施例は定量的多型解析方法の実施例である。

#### 実施例33

本発明の蛍光消光プローブ: プローブEu47FおよびEu1392Rの調製

(5-1) 蛍光消光プローブEu47Fの合成

(5')CITAACACATGCAAGTCG(3')(I=inosine)の塩基配列をもつデオキシリボオリゴヌクレオチドの5'末端のリン酸基に、下記のようにしてボデピーFLで標識した蛍光消光プローブEu47FをDNA合成機ABI 394 (Perkin Elmer社製、米国) で合成した。

#### 【0268】

(5-2) Eu1392Rの合成

(5')TTGTACACACCGCCCGTCA(3')の塩基配列をもつデオキシリボオリゴヌクレオチドを合成した。

#### 【0269】

上記のデオキシリボオリゴヌクレオチドの5'末端のリン酸基に、 $-(CH_2)_6-NH_2$ を結合したものをメドランド・サーティファイド・レージント・カンパニー社(Midland Certified Reagent Company、米国)から購入した。更に、モレキュラープローブ(Molecular Probes)社からフロオ・リポーターキット(FluoReporter Kits)F-6082(ボデピーFLのプロピオン酸サクシニジルエステル(BODIPY FL propionic acid succinidyl esters)の他に、当該化合物をオリゴヌクレオチドのアミン誘導体に結合させる試薬を含有するキット)を購入した。当該キットを前記購入の前記デオキシリボオリゴヌクレオチドに作用させて下記のボデピーFLで標識した本発明の上記蛍光消光プローブを合成した。

#### 【0270】

なお、前記合成物の精製は以下のように行った。

合成物を乾固し乾固物を得た。それを0.5M  $Na_2CO_3/NaHCO_3$ 緩衝液(pH9.0)に溶解した。当該溶解物をNAP-25カラム(ファルマシア社製)でゲルろ過を行い、未反応物を除去した。さらに逆相HPLC(B gradient:15~65%、25分間)を以下の条件で行った。そして、溶出するメインピークを分取した。分取した画分を凍結乾燥して、最初のオリゴヌクレオチド原料2mMより目的物を50%の収率で得た。

#### 【0271】

逆相クロマトグラフィーの条件:

溶出溶剤A: 0.05N TEAA 5% $CH_3CN$

溶出溶剤B(グラジエント(gradient)用): 0.05N TEAA 40% $CH_3CN$

カラム: CAPCELL PAK C18; 6×250mm

溶出速度: 1.0ml/min

温度: 40℃

検出: 254nm

#### 【0272】

## 実施例 3 4

## (6-1) 大腸菌 JM109 株の培養

53 培地 (組成: カゼインペプトン (カゼインのトリプシン消化物)、10 g; 酵母エキス、5 g; グルコース、5 g; 食塩、5 g; 蒸留水、1000 mL) を用いて大腸菌 JM109 株を培養した (培地 50 mL / 250 mL 容コニカルフラスコ、37℃、12 時間、振とう培養)。そして、培養液から菌体を集めた (遠心分離 10,000 rpm、5 分、蒸留水で 2 回洗浄)。

## 【0273】

## (6-2) 16S rRNA の cDNA の調製

菌体から、SOGEN キット (ニッポンジーン社) を用いて全 RNA を本キットのプロトコルに従って抽出した。

その後、BcaBEST<sup>TM</sup> RNA PCR キット (宝酒造株式会社) を用い、本キットのプロトコルに従って、前記抽出液について、16S rRNA を対象とした増幅と逆転写反応 (RT-PCR) を公知の通常の条件で行った。その際、前記の本発明の蛍光消光プローブ Eul392R をプライマーとして用いた。続いて、RNA を Rnase H により分解し (30℃、20 分)、16S rRNA 遺伝子の純粋な cDNA を得た。cDNA 濃度を OliGreen<sup>®</sup> ssDNA Quantitation キット (Molecular Probes) を使用して測定した。

## 【0274】

## 実施例 3 5

## (7-1) 定量的 PCR、データ解析および cDNA の検量線の作成

前記 cDNA 溶液について、本発明の蛍光消光プローブ Eu47F をフォワードプライマーとして、Eu1392R をリバースプライマーとして用い、リアルタイムモニタリング定量的 PCR 反応を行った。

リアルタイムモニタリング定量的 PCR 装置として、LightCycler<sup>TM</sup> Sytem (ロシュ・ダイアグノスティックス株式会社、ドイツ) を使用し、手順書記載の手順に従って反応を行った。なお、DNA ポリメラーゼとして TaKaRaTaq<sup>TM</sup> (宝酒造株式会社) を使用した。

## 【0275】

PCRは次のコンポーネントで行った。

大腸菌 cDNA	1.0 $\mu$ l (最終濃度 $10^3 \sim 10^6$ コピー)
プライマー溶液	4.0 $\mu$ l (最終濃度 0.1 $\mu$ M)
<u>TaKaRaTaq<sup>TM</sup></u>	10.0 ( $\mu$ l 0.5 Units)
ミリQ純水	5.0 $\mu$ l
全容量	20.0 $\mu$ l

なお、cDNAは、図30の注に示される実験区のコピー数で実験を行った。  
MgCl<sub>2</sub>の最終濃度は2mMであった。

#### 【0276】

反応条件は次の如くであった。

変性(denaturation)反応初期:	95℃、60秒
再:	96℃、10秒
アニーリング(annealing)反応:	50℃、5秒
DNA伸長反応:	72℃、60秒

測定条件は次の如くであった。

励起光	:	488nm
測定蛍光色	:	530nm

#### 【0277】

前記の条件でリアルタイムモニタリング定量的PCRを行って、各サイクルの蛍光強度を実測した。その実測値を本発明のデーター解析方法に従って解析した。  
すなわち、次の過程でデータを処理した。

(a) 各サイクルにおける増幅した核酸が蛍光色素で標識された核酸プライマーとハイブリダイズしたときの反応系の蛍光強度値(すなわち核酸伸長反応終了時(72℃)の蛍光強度値)を、増幅した核酸が核酸プライマーとハイブリダイズしたものが完全に解離したときの反応系の蛍光強度値(すなわち核酸熱変性反応終了時(96℃)の蛍光強度値)で割る補正演算処理過程、すなわち、実測の蛍光強度値を[数式1]で補正した。

#### 【数8】

$$f_0 = f_{\text{hyb. n}} / f_{\text{den. n}} \quad \dots \text{【数式1】}$$

〔式中、 $f_n$ =サイクルの蛍光強度の補正值、 $f_{nys, n}$ =各サイクルの72℃の蛍光強度値、 $f_{den, n}$ =各サイクルの96℃の蛍光強度値〕

#### 【0278】

(b) 各サイクルにおける〔数式1〕における補正演算処理値を〔数式3〕に代入し、各サイクルにおける各サンプル間の蛍光消光率を算出する演算処理過程、すなわち、下記の〔数式10〕で演算処理する過程、

#### 【数9】

$$F_n = f_n / f_{as} \quad \dots \text{〔数式10〕}$$

〔式中、 $F_n$ =各サイクルの演算処理値、 $f_n$ =〔数式1〕で得られた各サイクルの値、 $f_{as}$ =〔数式1〕で得られた値で、サイクル数が25回目のものである。〕

〔数式10〕は〔数式3〕において、 $a=25$ とした場合におけるものである。

#### 【0279】

(c) 前記(b)の過程で得られた各サイクルの演算処理値を〔数式6〕による蛍光強度の変化率(減少率または消光率)の対数値を演算処理をする過程、すなわち、下記の〔数式11〕で演算処理する過程、

#### 【数10】

$$\log_{10} \{ (1 - F_n) \times 100 \} \quad \dots \text{〔数式11〕}$$

〔式中、 $F_n$ =〔数式10〕で得られた値〕。

〔数式11〕は〔数式6〕において、 $b=10$ 、 $A=100$ とした場合におけるものである。

#### 【0280】

上記の結果を図30に示した。

図30は、前記(a)、(b)、(c)の過程で計算された値を、サイクル数に対してプロットし、印字したものである。

#### 【0281】

次に、図30のグラフを基に、次の(d)および(e)の過程で処理した。

(d) 前記(c)の過程で処理されたデータの内、0.2をスレッショールド(threshold)し、その値に達したサイクル数を計算する過程。

(e) 前記 (d) の過程で計算した値を X 軸に、反応開始前のコピー数を Y 軸にプロットしたグラフ、すなわち大腸菌 cDNA の検量線 (図 3 1) を作成する過程。

図 3 1 は、本発明の定量的 PCR 方法で得られるデータを、本発明のデータ解析方法、すなわち、(a)、(b)、(c)、(d)、(e) の過程で処理した最終結果である。図 3 1 から未知コピー数の核酸試料について反応開始前のコピー数を精度よく求めることができることが分かる。

【0282】

#### 実施例 3 6

##### (8-1) 多型系 (複合微生物系) の構築

(表 5 に示した 10 種類の細菌菌株を DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) から購入し、前記の 5 3 培地を用いて各々の菌株について別個に培養した。培養条件は前記大腸菌の場合と同様である。各々の培養液から菌体を集めた (遠心分離 10,000 rpm、10 分、蒸留水で 2 回洗浄)。各々の菌体について、前記と同様にして SOGEN キット (ニッポンジーン社) を用いて全 RNA を抽出した。

【0283】

表 8

菌株No.	DSMZ No.	HhaI断片 (bp)	T-RFLP から求めた モル構成率 (%)	定量コピー数	定量コピー数/ 初期添加コピー数
1	65	22	9.5	27400	0.91
2	3138T	43	10.9	31400	1.05
3	12778	52	9.7	27900	0.93
4	20530	104	9.4	27100	0.90
5	50108	168	10.4	30000	1.00
6	20152	332	9.3	26800	0.89
7	43879	404	9.7	27900	0.93
8	20579	432	9.9	28500	0.95
9	5078	531	10.4	30000	1.00
10	43673	626	10.8	31100	1.04

## 【0284】

- 1:Paracoccus pantotrophus
- 2:Sphingomonas natatoria
- 3:Bdellovibrio stolpii
- 4:Microbacterium imperiale
- 5:Pseudomonas fluorescens
- 6:Agromyces medislanum
- 7:Cellulomonas cellulans
- 8:Brevibacterium liquefaciens
- 9:Leminorella grimontii
- 10:Rhodococcus luteus

## 【0285】

その後、前記の大腸菌の場合と同様にして、それぞれの菌株の16S rRNA遺伝子の純粋なcDNAを得た。得られた10菌株各々のcDNA濃度を前記の大腸菌の場合と同様にして測定した。cDNA濃度の判明した溶液について、蒸留水にて300,000 copy/ $\mu$ Lとなるように希釈した。10菌株分について、希釈液を当量ずつ混合したものを複合微生物系すなわち多型系（以下、多型系という。）とした。この多型系には、10菌株分のcDNAがそれぞれ300,000 copy/ $\mu$ Lの濃度で含まれているので、全体として、3,000,000 copy/ $\mu$ Lの濃度cDNAが含有していることになる。

#### 【0286】

##### （8-2）リアルタイムモニタリング定量的PCR

前記多型系のcDNAについて、本発明の蛍光消光プローブEu47FおよびEu1392Rを菌株共通のプライマーとして用いて、前記大腸菌と同様にしてリアルタイムモニタリング定量的PCRを行った。

多型系のサンプルを、絶対量で300,000 copy/20 $\mu$ L（反応液全体20 $\mu$ L）となるように反応液に添加した。多型系のリアルタイムモニタリング定量的PCRは、蛍光強度の減少が観察され、かつ遺伝子の指数関数的増幅期である22サイクル数で反応を停止させた（図30参照）。スレッシュホールドを、 $\log R_n$ （蛍光消光率）=0.2と設定したときの、多型系について行ったリアルタイムモニタリング定量的PCRの反応液のcDNAのコピー数は288,000コピーであった（図31参照）。初期添加量すなわち理論値は300,000コピーであるから、本発明の方法によって作成された検量線は良好な定量性を示すことが確認された。

#### 【0287】

##### 実施例37

##### 多型解析

##### （9-1）T-RFLPによる解析

前記のようにしてPCR反応を行った後、増幅産物をカラム（MicroconPCR、Millipore Corporation, Bedford, MA, USA）を用いて精製した。精製物を制限酵素Hha1（認識部位：GCG/C、/＝切断個所）でO/N（一晚）処理した。

処理終了後、切断断片のみをカラム (Microcon及びMicropure-EZ、Millipore Corporation, Bedford, MA, USA) で精製した。制限酵素処理後の各菌株の cDNA 断片の大きさは、表 8 に示した。

### 【288】

カラム精製を施した cDNA 溶液について、加熱変性処理を行った後、シーケンサー (ABI PRISM 310、PE Applied Biosystems) にて T-RFLP 解析を行った。そのピークパターンを図 32 に示した。各ピークを濃度が既知である標準 BODIPY FL 修飾断片を用いて定量した。各ピークのモル構成率を求めた結果、すべてのモル構成率は、9.4~10.8 の範囲に収まっており、PCR 増幅効率の極端な差異認められなかった (表 8 参照)。定量的 PCR で求めた全 cDNA のコピー数にモル構成率を掛け、それぞれの菌株の初期の cDNA のコピー数を求めた (表 8 参照)。定量により求めたコピー数/初期添加コピーは 0.89~1.04 (表 8 参照) であった。よって、本方法により多型系における多型の初期コピーを正確に定量できることが判明した。

### 【0289】

実施例 38 蛍光発光プローブをプライマーとして用いた (以下、蛍光発光プライマーという。) リアルタイム定量的 PCR 法と本方法を適用した定量的多型解析法の実施例

蛍光発光プライマーを用いたリアルタイム定量的 PCR 法とこのリアルタイム定量的 PCR 法を利用した定量的多型解析法の実施例について説明する。

### 【0290】

#### 1) 実験方法および条件

##### <人工複合微生物系 (テンプレート) の調製>

人工複合微生物系を調製し、これをモデル系として定量的多型解析法の有効性を証明した。実験は、Table 9 に示した 10 種の微生物を DSM より購入した。各々の菌株を 53 培地を用いて別々に培養した。培養液から菌体を集菌し、キット試薬 ISOGEN (ニッポンジーン、日本) より Total DNA をプロトコルに従い抽出した。その後、Eu47F (CITAACACATGCAAGTCG, I=inosine)、Eu1392R (TTGTACACACCGCCCGTCA) をプライマーとして、16sRNA 遺伝子を増幅対象として

P C R反応を行った。得られた10種の16SrRNA遺伝子増幅産物をPicoGreen<sup>R</sup> dsDNA Quantitation Kit (Molecular Probes)にて定量した後、滅菌蒸留水にて300,000 copies/mlとなるようそれぞれ希釈した。これらを等量混合したものを、モデル人工複合微生物系とした。このモデル人工複合微生物系には10種の微生物の16SrRNA遺伝子増幅産物がそれぞれ30,000 copies/mlの濃度で含まれており、トータルの16SrRNA遺伝子増幅産物濃度は300,000 copies/mlとなる。

#### 【0291】

<本発明の蛍光発光プライマーを用いたリアルタイム定量的P C R実験方法>

上記の人工複合微生物系(16S rRNA遺伝子混合サンプル)を対象として、Texas red, DABCYL2重修飾蛍光発光プライマーを用いた定量的P C Rを行った。共通プライマーとしてはEu47F-modi(CITAACACATGCAAGTCG, I=inosine)、Eu1392R(TTGTACACACCGCCCGTCA)を使用した。Eu47F-modiは、Eu47Fと塩基配列は同様であるが、5'末端から9番目のTがTexas red、9番目のTがDABCYL修飾されている。Texas redおよびDabcylの修飾方法は、実施例7と同様であった。定量的P C R装置としては、iCycler(バイオラッド(BIO-RAD)社製)を用いた。最初のDenatureは95℃で60秒間行い、P C RサイクルはDenature=95℃/60秒, annealing=50℃/60秒, extention=72℃/70秒の条件にて行った。P C R反応は、初期遺伝子構成比が崩れないように(P C Rバイアスがかからないように)指数関数的な増幅領域でストップさせた。Primer濃度は、Eu47F、Eu1392R共に最終濃度で各0.1 μMとした。DNA polymeraseはTaKaRa TaqTM(宝酒造)を0.5 Units/20 μlの濃度で使用した。Mgイオン濃度は2 mMとした。d N T Pは最終濃度で各2.5 mMとなるよう添加した。AntiTaq body(クローンテック社製)を使用し製造者の指導書に則り、Hot startを行った。検量線作成のための標準サンプルとしては、E. coliの16s rDNA遺伝子増幅産物を用いた。E. coliの16s rDNA遺伝子増幅産物調製は、前記人工複合微生物系と同様の方法で行った。検量線作成後、人工複合微生物系の定量を行った。人工複合微生物系の遺伝子量は、絶対量で300,000 copy/20 μl(20 μl=全量)となるよう調整した。蛍光測定は各サイクルのdenature後、annealing後に一回ずつ行った。蛍光発光率は、蛍光消光率と同様、annealing後(ハイブリダイゼーション

時)の蛍光強度をdenature後(解離時)の蛍光強度で補正することで求めた。

# 【0292】

具体的な計算式は

$$F_n = \{ (f_{\text{hyb}, n} / f_{\text{den}, n}) / (f_{\text{hyb}, n'} / f_{\text{den}, n'}) \} \times 100$$

$F_n$  =  $n$ サイクル時の蛍光発光率

$f_{\text{hyb}, n}$  =  $n$ サイクルにおけるannealing(ハイブリダイズ)時の蛍光強度

$f_{\text{den}, n}$  =  $n$ サイクルにおけるdenature(解離)時の蛍光強度

$f_{\text{hyb}, n'}$  = 増幅産物由来の蛍光発光が起こる前のサイクル( $n'$  サイクル)におけるannealing(ハイブリダイズ)時の蛍光強度

$f_{\text{den}, n'}$  = 増幅産物由来の蛍光発光が起こる前のサイクル( $n'$  サイクル)におけるdenature(解離)時の蛍光強度

# 【0293】

<T-RFLPによる解析>

リアルタイム定量的PCR反応終了後、増幅産物の精製をカラム(Microcon PCR, Millipore Corporation Bedford社, MA, USA)にて行い、HhaI(認識部位: GCG /C, / = 切断箇所)にて制限酵素処理を行った。この制限酵素断片を含む溶液を、加熱変性処理を行った後、シーケンサー(ABI PRISM 310, PE Applied Biosystems)にてT-RFLP解析を行った。その後、各制限酵素断片を同鎖長の蛍光発光プローブを標準物質として定量した後、各ピークのもル構成率を求めた。

# 【0294】

## 2) 結果

<蛍光発光プライマーを用いたリアルタイム定量的PCR結果>

Fig. 33, Fig. 34. に結果を示す。Fig. 33. から分かるように、蛍光発光プライマーを用いて増幅産物のモニタリングが可能であることが示された。また、スレッシュホールド(thresh hold)値( $\log F_n$ (蛍光発光率) = 1.6)に達するのに必要であったサイクル数と初期に(innitial)添加したDNA量との関係をFig. 34に示した。この図から分かるようにサイクル数と初期添加のコピー数とは直線関係にあることが分かる。従って、この図からスレッシュホールド値に

達したサイクル数より、初期の標的遺伝子の定量が正確に行えることが示唆された。人工複合微生物系では、対数的増幅が見られるサイクル数（23サイクル）で停止させた（Fig. 33参照）。Fig. 34として示した検量線より、人工複合微生物系の16S rRNAコピー数は、約296,000 copiesと定量された。初期添加量は300,000 copiesであったことから、本法の良好な定量性が確認された。

#### 【0295】

##### < T-RFLPによる解析結果 >

リアルタイム定量的PCRの増幅産物をT-RFLP方法で解析し、制限酵素断片をそれぞれ定量した結果、全てのピークのモル構成率は、9.5～10.6の範囲内であり、16S rRNA遺伝子種によるPCR増幅効率の差異は認められなかった（Table 9参照）。定量的PCRで求めた16s rRNA遺伝子のトータルコピー数にモル構成率をかけ、それぞれの構成微生物の初期16s rRNA遺伝子のコピー数を求めた（Table 9参照）。定量より求めたコピー数／初期添加コピー数は0.94～1.05（Table 9参照）であることより、本法の人工複合微生物系の混合遺伝子の初期コピー数の定量（標的核酸の定量）は精度がよいことが証明された。

#### 【0296】

【表9】 人口複合微生物系の混合遺伝子のT-RFLP方法による解析結果

Table9: T-RFLP結果 (発光プライマー) <sup>「発光」</sup> <sup>「プライマー」</sup>

微生物名	DSM No.	HhaI 断片長 (bp)	T-RFLPから求め た構成率	定量コピー数	定量コピー数/ 初期添加コピー
Paracoccus pantotrophus	65.	22	9.50 %	28120	0.94
Shingmonas natatoria	3183T.	43	10.10 %	29896	1.00
Bdellovibrio stolpii	12778	52	9.90 %	29304	0.98
Microbacterium imperiale	20530	104	9.60 %	28416	0.95
Pseudomonas fluorescens	50108	168	9.70 %	28712	0.96
Agromyces medislanum	20152	332	10.10 %	29896	1.00
Cellulomonas cellulans	43879	404	9.80 %	29008	0.97
Brevibacterium liquefaciens	20579.	432	10.40 %	30784	1.03
Leminorella grimontii	5078	531	10.30 %	30488	1.02
Rhodococcus luteus	43673	626	10.60 %	31376	1.05

## 【0297】

実施例39 蛍光発光プローブを用いたリアルタイム定量的PCR法の実験例

従来技術のプライマーと本発明の蛍光発光プローブを用いて定量的PCR法を行い、当該プローブで増幅産物をリアルタイムモニタリングすることを基本原理とするリアルタイム定量的PCR法の実施例について説明する。

## 【0298】

## 1) 実験方法および条件

## &lt;テンプレートDNAの調整&gt;

Paracoccus denitrificans DSM 413のゲノムDNAをDNeasy™ Tissue Kit (QIAGEN GmbH 社, Hilden, Germany) を用いて抽出後、E10F (AGAGTTTGATCCTGGCTCAG: 蛍光修飾なし)、E140R (GGTTACCTTGTTACGACTT) のプラ

イマーセットを用い、通常のPCRにて、16S rRNA遺伝子を増幅した。PCR増幅産物をPico Green dsDNA Quantitation Kit (Molecular Probes Inc.)を用いてそれぞれ定量した後、16S rRNA遺伝子を6 ng/ $\mu$ l含む溶液を調整した。

#### 【0299】

<その他の条件>

蛍光発光プローブ配列は、5' CTAATCCTTT-(Texas red) GGCGAT-(DABCYL) AAATC 3' であり、5'末端からの9番目のTをTexas red修飾、5'末端からの15番目をTをDABCYL修飾したものを使用した。その修飾方法は実施例7と同様である。また、当該プローブの3'末端は、3'末端からの伸長を阻害されるようにリン酸化された。Forward, Reverseプライマーは通常のPCRで使用したものと同一のものを用いた(E10F, E1400R) (すなわち、蛍光色素で修飾されていないプライマー)。リアルタイムPCR装置はiCycler (バイオラッド)を用いた。

#### 【0300】

PCR条件は、通常のPCR、リアルタイム定量的PCR法ともに、それぞれ1st denatureは95℃, 120sec、denatureは95℃, 60sec、annealは56℃, 60sec、extensionは72℃, 70secの条件である。Mgイオン濃度は2mMとした。dNTPは最終濃度で各2.5mMとなるよう添加された。TaqポリメラーゼとしてGene Taq (日本ジーン)を用いた。プライマーは、通常のPCR方法、リアルタイム定量的PCR法ともに、最終濃度で100nM添加した。本DNA溶液は、標準テンプレート溶液として用い、0.6pg~6 ng/reaction となるよう添加した。テンプレートとして、上記の方法で調整したParacoccus denitrificans DSM 413由来の16S rRNA遺伝子増幅産物を用い、0.6pg~6 ng/reaction となるよう反応系に添加した。蛍光発光プライマーは50nM添加した。蛍光測定は各サイクルのdenature後、annealing後に一回づつ行った。蛍光発光率は、実施例38と同様の方法で求めた。

#### 【0301】

##### 2) 結果

蛍光発光プローブによる増幅産物のリアルタイムモニタリングした結果をFig. 35に示した。この図から、蛍光発光プローブを用いて増幅産物をモニタリングす

ることが可能であることが分かった。また、スレッシュホールド値 ( $\log F_n$  (蛍光発光率) = 1.8) に達するのに必要であったサイクル数と初期添加 DNA 量との関係を Fig. 35 に示した。この図から分かるようにサイクル数と初期添加のコピー数とは直線関係にあることが分かった。なお、この時の、相関係数は  $R^2=0.9993$  であった。従って、この図からスレッシュホールド値に達したサイクル数より、初期の標的遺伝子の定量が正確に行えることが分かった。

以上の結果より、蛍光発光プローブを用いたリアルタイム定量的 PCR 法により初期標的核酸 (増幅前に存在した標的核酸量) の測定が可能であることが証明された。

### 【0302】

**実施例 40** 蛍光発光プローブあるいは蛍光消光プローブを用いた 1 塩基多型の検出

蛍光発光プローブあるいは蛍光消光プローブを用いて、解離曲線より一塩基多型を検出する方法について、具体的実施例をあげて説明する。

### 【0303】

#### 1) 実験方法

蛍光発光プローブは、実施例 39 で使用したのと同じ蛍光発光プローブを用いた。蛍光消光プローブは、蛍光発光プローブと同様の配列で、5' 末端が BODIPY FL で修飾されたものを用いた。{(BODIPY FL)-5' CTAATCCTTTGGCGATAAATC 3'}。修飾方法は実施例 8 と同様である。ターゲットは上記蛍光発光プローブおよび、蛍光消光プローブと 100% 相補的な配列 ((5') GATTTATCGCCAAAGGATTAG (3')) と、相補的な配列であるが、5' 末端から 10 番目の C が T に置換された一塩基多型を含む配列 ((5') GATTTATCGTCAAAGGATTAG (3')) を用いた。プローブは最終濃度 100 nM 添加した。合成ターゲット DNA は最終濃度 400 nM 添加した。ハイブリダイゼーション溶液の組成は実施例 12 で使用したのと同様である。合成ターゲット DNA は、用意した 2 種類のターゲットの内、どちらか一方を使用した。実験は、予め上記の条件で調整した溶液を蛍光測定用チューブに添加し、これを 0.1 °C/sec で 30 °C から 80 °C まで昇温させ、その間蛍光測定を連続的に行った。

## 【0304】

この蛍光測定結果から、プローブとターゲットとの解離曲線を作成し、その解離曲線の違いから一塩基多型を含む配列を判別可能か評価した。実験装置として、iCycler（バイオラッド社）を用いた。蛍光フィルターは、蛍光発光プローブの蛍光検出にはバイオラッド社の提供しているTexas red 用の蛍光フィルターを、蛍光消光プローブの蛍光検出には同じくバイオラッド社の提供しているFITC用の蛍光フィルターを用いた。

## 【0305】

## 2) 結果

結果をFig. 36として示す。この図から、1塩基多型を含むターゲットとの解離曲線のT<sub>m</sub>値は、蛍光発光プローブ、蛍光消光プローブ共に100%相補的ターゲットとの解離曲線のT<sub>m</sub>値より約10℃低いことが分かった。これは、1塩基分の水素結合の有無がT<sub>m</sub>の差となって現れたことを示した。以上のことから、蛍光発光プローブあるいは蛍光消光プローブを用いることにより、1塩基多型を簡便に区別できることが証明された。

## 【0306】

## 実施例41 蛍光発光プローブを用いたDNAチップ

蛍光発光プローブを用いたDNAチップについて、具体的に実施例をあげて説明する。

## 【0307】

## 1) 実験方法

Table 10に示した配列の蛍光発光プローブを調製した。これらは、全てヒトのCYP21遺伝子の部分配列であり、プローブ配列中にSNPs部位を含んでいる。

プローブ名称は、the Whitehead Institute([http://waldo.wi.mit.edu/cvar\\_snps/](http://waldo.wi.mit.edu/cvar_snps/))のSNPsのID番号をそのまま利用した。合成法は以下の2点を除き、実施例7.と同様である。(1) 5'末端は、5'-Amino-Modifier C12 (Glen Research社製)を用いてアミノリンカーを導入した。(2) Texas redは、プローブ配列によってAmino-Modifier C6 dTだけでなく、Amino-Modifier C6 dC (グレンリサーチ社製)も用いて修飾した。プローブ配列およびTexas red

Dabcylのプローブ内の修飾位置はTable 10に示した通りである。標的核酸はTable 12に示したものを使用した。

【0308】

【表10】 使用した蛍光発光プローブ

使用した~~蛍~~光プローブ

70-7名	配列 (下線部がSNPs部位)	5'末端からの Texas red修飾位置 (5'末端塩基=0番目)	5'末端からの Dabcyl位置
WIAF-10544	5'CGCAGCCGAG CATGGAAGA 3'	6	12
WIAF-13038	5'CGCTGCTGCC CTCCGG 3'	5	11
WIAF-10600	5'AAGGGCACGT GCACATGGC 3'	9	15
WIAF-10579	5'CATCGTGGAG ATGCAGCTGA GG 3'	5	11
WIAF-10578	5'CCTGCAGCAT CATCTGTTAC CTCAC 3'	10	16

【表 1 1】 使用したターゲット核酸配列

ターゲット核酸配列

70-7名	配列	備考
No.1 100% match target	5'TCTTCCATGC TCGGCTGCG 3'	修飾なし
No.1 1 mismatch target	5'TCTTCCATGC <u>T</u> CGGCTGCG 3'	修飾なし、 下線部ミスマッチ部
No.2 100% match target	5'CCGGAGGGCA GCAGCG 3'	修飾なし
No.2 1 mismatch target	5'CCGGAGG <u>A</u> CA GCAGCG 3'	修飾なし、 下線部ミスマッチ部
No.3 100% match target	5'GCCATGTGCA CGTGCCCTT 3'	修飾なし
No.3 1 mismatch target	5'GCCATGTGCA <u>A</u> GTGCCCTT 3'	修飾なし、 下線部ミスマッチ部
No.4 100% match target	5'GCCTGCCACG AGGCTCTCC 3'	修飾なし
No.4 1 mismatch target	5'GCCTGCCAC <u>C</u> AGGCTCTCC 3'	修飾なし、 下線部ミスマッチ部
No.5 100% match target	5'GTGAGGTAAC AGATGATGCT GCAGG 3'	修飾なし
No.5 1 mismatch target	5'GTGAGGTAAC AG <u>T</u> TGATGCT GCAGG 3'	修飾なし、 下線部ミスマッチ部

## 【0309】

## &lt; DNAチップの調製 &gt;

スポティングは、各プローブ溶液につき、1スポットずつ行った。これ以外のDNAチップの調製法は、前記の蛍光消光プローブを用いたDNAチップの調製方法と同様である。

スライドガラス上に固定化された本発明のプローブは、標的核酸にハイブリダイズしないときTexas redは蛍光が消光しているが、ハイブリダイズしているときは蛍光の発光が、ハイブリダイズしないときのものよりも著しく増加する。

## 【0310】

## &lt; SNPs の検出測定方法 &gt;

各々100  $\mu$ M濃度で含む5種類の100%matchターゲット混合溶液 {50 mMのTE緩衝液 (pH:7.2) 使用} を、上記のごとくに調製したDNAチップの上にのせ

た。 各々100  $\mu$ M濃度で含む5種類の1 mismatchターゲット混合溶液も同様に調製し、100%matchターゲット混合溶液をのせたものとは別のDNAチップの上にのせた。 これらをカバーガラスで覆い、標的核酸が漏れないようにマニキュアにてカバーガラスをシールした。 従って、本試験では計2枚のDNAチップを調製した。 これらのチップについてそれぞれ、温度を変化させながら連続的に蛍光観察を行い、ターゲットとの解離曲線を作成した。

### 【0311】

#### <測定装置>

検出測定のための装置類は前記図13に示したものと同様である。

### 【0312】

#### 2) 実験結果

実験結果をFig. 37に示した。 図から、温度が低くなるに従い、全てのプローブで蛍光強度が上昇していることが分かる。 これは、蛍光発光プローブがターゲット配列とハイブリダイズしたことを示している。 従って、本発明の方法により、本発明のプローブと標的核酸との解離曲線を簡便にモニタリングすることができる事が示された。 また、標的核酸と100%マッチするプローブと一塩基ミスマッチするプローブとの $T_m$ 値の差は、本検討の場合10℃前後であるので、解離曲線から両者を容易に識別することができた。 すなわち本発明のDNAチップを使用することにより複数種のSNPsの解析が同時に実施できることを本実験は示した。

### 【0312】

実施例42 蛍光発光プローブ、蛍光消光プローブを固定化したDNAチップ上での遺伝子増幅と増幅産物のリアルタイム検出

✓ 蛍光発光プローブ、<sup>72</sup>蛍光消光プローブを固定化したDNAチップ上に於いて遺伝子増幅を行うと共に増幅産物をリアルタイムモニタリングする手法について、具体的実施例をあげて説明する。 また、増幅された遺伝子と蛍光発光プローブと蛍光消光プローブとの解離曲線から、SNPsの検出を行った。

### 【0313】

#### 1) 実験方法

## (1) 蛍光発光プローブ

蛍光発光プローブおよび蛍光消光プローブを、Table 12に示した。これらは、実施例41で使用したのと同じ配列である。蛍光発光プローブは、3'末端がリン酸化されたものを用いた。そらの合成法は、実施例41と同様である。プローブ配列およびTexas redとDabcylのプローブ内の修飾位置はTable 10に示した通りである。

## 【0314】

## (2) 蛍光消光プローブ

蛍光消光プローブの配列は、蛍光発光プローブの配列と同様である。蛍光消光プローブの5'末端は、5'-Amino-Modifier C12 (グレンリサーチ社製)を用いてMMTアミノリンカーを導入した。また、3'末端塩基はAmino-Modifier C6 dC (グレンリサーチ社製)を用いて、TFAアミノリンカーを導入した。保護基であるTFAを脱保護した後、アミノリンカーを介してBODIPY FL (Molecular probes)修飾した。また、蛍光消光プローブは3'末端がリン酸化されたものである。標的核酸はTable 11に示したものを使用した。その他の詳細な精製法や修飾法は、実施例8.と同様であった。

## 【0315】

## (3) プライマー

フォワードプライマーとして5' CTTGGGGGGGCATATCTG 3'である配列を用い、リバースプライマーとして5' ACATCCGGCTTTGACTCTCTCT 3'を用いた。このプライマーセットは、ヒトのCYP21遺伝子の一部(2509bp)を増幅する事が可能である。蛍光発光プローブと蛍光消光プローブは、SNPsを含まない増幅産物に対し100%相補的な配列を有するものである。よって、増幅産物が増えるに従い、Table 12として示した蛍光発光プローブおよび蛍光消光プローブの蛍光強度の変化量は増大する事が予想された。

## 【0316】

## &lt;DNAチップの調製&gt;

スライドガラスには、各プローブ溶液につき、1スポットずつスポッティングした。これ以外のDNAチップの調製法は、消光プローブを用いたDNAチップ

の調整法と同様である。

### 【0317】

スライドガラス上に蛍光発光プローブが固定化された場合、プローブが標的核酸にハイブリダイズしないときTexas redの蛍光が消光しているが、ハイブリダイズしているときは蛍光の発光が、ハイブリダイズしないときのものよりも著しく増加する。これとは逆に、スライドガラス上に蛍光消光プローブが固定化された場合、標的核酸にハイブリダイズしないときBODIPY FLは蛍光が発光しているが、ハイブリダイズしているときは、ハイブリダイズしないときよりも著しく蛍光が消光する。

### 【0318】

#### <リアルタイムモニタリングPCRの方法>

実施例28で用いたヒトゲノムをテンプレートとして、前述のプライマーを用いDNAチップ上でPCRを行い、PCR増幅産物を固定化された蛍光発光プローブまたは蛍光消光プローブにて検出した。実験は、前記図13に示した装置を用いて行われた。蛍光発光プローブと蛍光消光プローブが固定化されたDNAチップ上に、プライマー、テンプレート、Taq polymerase, dNTP,  $MgCl_2$ などを含む溶液をのせた。この溶液が漏れないようにカバーガラスで覆い、マニキュアにてカバーガラスをシールした。このチップを温度制御プログラムを組み込んだ透明加熱板にのせ、チップ上でPCR反応を行わせた。増幅された産物は、固定化された蛍光発光プローブと蛍光消光プローブの蛍光変化量を図13に示した顕微鏡にて追うことで、リアルタイムで検出された。

### 【0319】

最初のDenatureは95℃で120秒間行い、PCRサイクルはDenature=95℃/60秒, annealing=60℃/60秒, extention=72℃/120秒にて行った。Primer濃度は、フォワード、リバース共に最終濃度で各0.5  $\mu M$ とした。テンプレートは、1.5ng/ $\mu l$ の最終濃度で添加した。DNA polymeraseとしてGene TaqTM（日本ジーン）を0.5 Units/20  $\mu l$ の濃度で使用した。Mgイオン濃度は2mMとした。dNTPは最終濃度で各2.5mMとなるよう添加した。

### 【0320】

### < 解離曲線の作成 >

固定化された蛍光発光プローブおよび蛍光消光プローブとPCR増幅産物との解離曲線を、実施例41と同様の方法で作成し、SNPsの検出を行った。

【0321】

#### 2) 結果

実験結果をFig. 38に示した。図からサイクル数の増加に伴い、全てのプローブで蛍光変化量が上昇していることが分かる。従って、本発明の方法により、遺伝子増幅とその増幅産物のリアルタイム検出が同時に行えることが示された。増幅産物と各プローブとの解離曲線を作成した結果をFig. 39に示す。図から温度が低くなるに従い、全てのプローブで著しい蛍光変化が見られた。これは、蛍光発光プローブおよび蛍光消光プローブがターゲット配列とハイブリダイズしたことを示した。このように、本発明のプローブと標的核酸との解離曲線を簡単にモニタリングすることができる事が分った。また、増幅産物とWIAF-10600の蛍光発光プローブおよび蛍光消光プローブとの解離曲線は、実施例41で得られたミスマッチを含まない人工合成ターゲットとWIAF-10600プローブとの解離曲線とほぼ一致しており、今回テンプレートとして使用したヒトゲノムは、WIAF-10600のプローブ配列と100%相補的であることが示された。また、増幅産物とWIAF-10578の蛍光発光プローブおよび蛍光消光プローブとの解離曲線は、実施例41で得られたミスマッチを含む人工合成ターゲットとWIAF-10578プローブとの解離曲線とほぼ一致しているので、今回使用したヒトゲノムは、WIAF-10578のプローブ配列に対しミスマッチを含むことが示された。この様に、本発明のDNAチップを使用することにより、遺伝子増幅を行った後に増幅された産物における複数種のSNPsの解析を同時に実施できることが分かった。

【0322】

發光

プローブ名	プローブ種	配列	5'末端からの Texas red修飾位置 (5'末端塩基=0番目)	5'末端からの Dabcyl 位置
WIAF- 10600-No. 1	蛍光発 光プローブ	5'AAGGGCACGT GCACATGGC 3'	6	12
WIAF- 10578-No. 2	蛍光発 光プローブ	5'CCTGCAGCAT CATCTGTTAC CTCAC 3'	5	11
WIAF- 10600-No. 3	蛍光発 光プローブ	5'AAGGGCACGT GCACATGGC 3'	9	15
WIAF- 10579-No. 4	蛍光発 光プローブ	5'CCTGCAGCAT CATCTGTTAC CTCAC 3'	5	11